Some applications of post-column ion pair extraction detectors in HPLC

Roy, Irfan Michael

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SOME APPLICATIONS OF POST-COLUMN ION PAIR EXTRACTION DETECTORS IN HPLC

Thesis

Submitted by Irfan Michael Roy, B. Pharm, M.Pharm.

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Dedicated

to

my parents

Summary

In this thesis:

1). A phase separator designed by Dr T.M.Jefferies has been evaluated for use with an HPLC post-column ion-pair extraction system.

2). Various chromatographic columns have been examined for the HPLC of basic drugs and most suitable ones have been used for various applications.

3). The selectivity of an HPLC post-column ion pair extraction system has been compared with detection at 205 nm for selected drugs of abuse from urine.

4). The post-column system designed in this thesis has been utilised for the detection of cocaine and its major metabolites i.e benzoylecgonine and ecgonine methylester in urine. The method developed is proposed as a general screening method for the identification of cocaine abuse.

5). A solid phase extraction method has been developed using large capacity (300 mg) cartridges for the extraction of the above cocaine metabolites from urine which has increased the recovery of EME from 40% to over 80%.

6). Various fluorescent dyes were examined with regards to their optical properties and extraction constants for use as post-column ion-pairing agents.

Contents

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INTRODUCTION

Reversed phase high performance liquid chromatography (RP-HPLC) has gained wide popularity since its introduction in 1970, for the analysis of drugs in biological matrices, so that it has become a ubiquitous technique in bioanalytical laboratories. Although a large number of improvements have been made, for example in the field of column technology leading to efficient general purpose and speciality columns (Majors, 1989, 1992) and other hardware components of the system, RP-HPLC often cannot meet the demands of modern trace organic and biomedical analysis with regards to detector performance. This is because many compounds present in biological matrices such as metabolites, have lost chromophores or fluorophores due to metabolic processes and so are difficult to detect by the UV or fluorescence detectors normally used in HPLC. Moreover they are present in such small quantities (µg-ng) that their detection using bulk property detectors such as refractive index detectors is not possible.

Probably the best technique for the analysis of drugs and metabolites in biological fluids is RP-HPLC-MS, as it provides the flexibility of reversed phase chromatography plus structural identification by mass spectrometry. Unfortunately due the problems of interfacing LC with MS the cost places the technique out of the reach of many laboratories. Under such circumstances, the problems of detection of difficult compounds and metabolites can be solved by careful sample preparation and if need be, suitable derivatization techniques. One way of achieving enhanced and selective detection of these analytes is by the use of solid phase extraction cartridges (SPE) for sample preparation followed by post-column derivatization.

This thesis describes the use of SPE followed by post-column ion-pair extraction and detection to enhance detection and improve selectivity for some compounds of forensic interest and their metabolites which are present in biological samples and whose detection is a major problem. This chapter gives a general over-view of the techniques used in this thesis.

1.1 Sample preparation

Sample clean up is an essential part of any chromatographic procedure and this is especially important for biological samples as they contain many endogenous substances e.g. proteins in plasma, fatty acids and amino-acids in urine samples, which may interfere with subsequent steps prior to chromatography.

Sample preparation is done mainly to improve accuracy, increase column life, improve detectability (by pre-concentration), and remove sample components which may interfere with the analysis. The procedure for sample preparation depends upon the nature of the sample. For many bioanalytical procedures urine is the preferred sample as it can be easily collected in large amounts, the concentrations of most drugs and metabolites are relatively high in urine, and the protein content of urine samples is low (Lingeman and Tjaden 1990). In some cases e.g., drug monitoring and pharmacokinetic studies plasma samples are preferred. The disadvantage with urine samples is that they contain a large number of components (over 600). Sample preparation from biological samples is normally done by either liquid-liquid extraction or solid phase extraction.

1.1.1. Liquid-liquid extraction

Liquid-liquid extraction (LLE) is based on the distribution of the compound between two immiscible liquids. A large number of organic solvents is available. The ideal organic solvent should give the best extraction for the compounds of interest with minimal extraction of other unwanted components of the sample. The pH of the aqueous phase plays an important role as only unionised compounds can be extracted into organic solvents and most drug substances are ionisable molecules. Sometimes the addition of ion-pairing agents improves the extraction of hydrophilic molecules. The advantages of LLE include adjustable selectivity, applicability to fairly polar and non-polar analytes, and applicability to a large number of aqueous samples. The disadvantages include formation of emulsions causing sample losses, use of toxic and flammable solvents, necessity of evaporation which may cause degradation of the analyte, cost, and often it is time consuming and laborious process. LLE is mainly carried out "off-line" but can also be carried out "on-line". Technicon markets an on-

line system for LLE called FAST-LC (fully automated sample treatment).(Huber and Frei, 1988).

Due to these disadvantages and the need for automation, LLE is being superseded by solid phase extraction. However for many compounds LLE is still a preferred method in many laboratories. For some of the compounds covered in this work e.g quaternary ammonium compounds and the cocaine metabolite ecgonine methyl ester, sample preparation from biological fluids is routinely performed using LLE. (See Chapter 5 and Chapter 6).

1.1.2.Solid phase extraction (SPE)

This involves the use of a disposable cartridge which can either be packed manually with a suitable sorbent or purchased ready to use. Varian, Supelco and J.T.Baker are well known suppliers of these cartridges. Sorbent types are typically similar to the range of HPLC materials available except that larger particle size (e.g. 40 μm) are used. Some companies also market mixed mode phases for special use e.g Bond Elut 'Certify' is a mixture of hydrophobic and ion exchange material for basic drugs from Varian, 'Narc 1' for drugs of abuse and 'Narc 2' for cocaine and benzoylecgonine from Baker.

Solid phase extraction (SPE) or sorbent extraction is a physical process that involves a liquid and a solid phase. Here the solid phase has a greater attraction for the isolate (analyte) than the solvent in which the analyte is dissolved. As the sample solution passes through the sorbent bed, the analyte is retained on its surface while other components pass through the bed. Very selective extraction can result by choosing sorbents with an attraction for the analyte but not for other sample components (Analytichem Handbook).

To use these solid phase cartridges they first have to be wetted with a suitable solvent and then conditioned. The sample is loaded on to the cartridge in an appropriate conditioning solvent and washed with suitable solvents to remove impurities and other endogenous material not required in the analysis. The drug sample (s) is then eluted from the cartridge with a minimum amount of eluting solvent.

The disadvantage of solid phase extraction methods the lack of reproducibility between batches of cartridges. Some manufacturers claim that their batches are highly reproducible. However very little information is available in this respect.

The advantages of SPE systems are that they can be readily automated either by the use of robots (McDowall et al, 1989), or by the use of automatic systems such as AASP and ASPEC or even by column switching. In column switching a short column is used in place of the injection loop in the injection valve. With the valve in the load position, the total sample is transferred onto the loop column. The valve is then turned to the inject position and the components of interest are eluted onto the analytical column while the impurities are retained on the loop column. It can also be used to remove impurities while in load position.

However it must be pointed out here that although the current trend is towards SPE using either cartridges or discs, in some cases e.g forensic analysis using blood, or environmental analysis (dirty water samples), or in the case of analysis of an unknown substance, LLE is still the first method of choice as comprehensive extraction schemes can be designed for the isolation of acidic, basic and neutral compounds from 'messy' samples. Efforts to semi-automate LLE by using mechanical shakers can make the method less time consuming until better methods for sample preparation are found.

1.2.Derivatization in chromatography

Lingenman and Underberg (1990) have described derivatisation as a necessary evil. Danielson et al (1988) have listed 248 references covering the period 1978-1987 where derivatisation has been used in HPLC. Derivatisation in chromatography has been used for a long time, especially in TLC (thin layer chromatography) where after development the plate is sprayed with a suitable reagent and then examined visually or otherwise. The main reasons for derivatisation are:

1 Improvement of the detectability of the analyte.

2. Improvement of the resolution of closely related compounds during chromatography .

3. Improvement of selectivity for the solute in a complex matrix.
4. Improvement of the chromatographic behaviour of drugs
5. Prevention of decomposition of a drug during chromatography
6. Introduction of an additional clean up step.

Derivatisation provides the desired selectivity and sensitivity and may involve a chemical reaction to modify the compound. Under the right conditions, by selecting a suitable reagent, an appropriate derivative may be obtained. Ideally derivatisation should be rapid and quantitative with a minimum of by-products, and the excess reagents should not interfere with the compounds of interest, and /or be easily removed.

Ideally fluorescence derivatization is preferred over UV derivatization. Fluorescence is more sensitive and selective and unlike electrochemical derivatization is less affected by mobile phase conditions. Another problem with electrochemical detection is the contamination of metal electrodes when complex samples are analysed (deRuiter, 1989). Derivatisation may be carried out in pre- or post- chromatographic mode.

1.2.1 Pre-chromatographic derivatization

Pre-column derivatisation or pre-chromatographic derivatization is carried out before the sample is injected on to the column. In LC pre-column derivatization is carried out mainly to improve the detection of analytes e.g the derivatization of fatty acids using 4-bromomethyl 7-methoxy coumarin (Dunges, 1977) or the derivatization of amines using sodium napthaquinone-4-sulphonate (Farrell and Jefferies, 1983). However pre-column derivatization in LC can also improve the chromatographic properties of the analyte e.g. increase the hydrophobicity of a poorly retained analyte in reversed phase HPLC (Hostettmann and McNair, 1976), improve separation, avoid decomposition of analyte or decrease the volatility of low molecular weight compounds (Lingenman and Underberg, 1990). According to these authors GC derivatization techniques e.g acylation, alkylation and silylation can also be used in LC, although some reactions will be more suitable for LC conditions than others. Reagents or derivatives which are sensitive to moisture will generally be difficult to use in

RP-HPLC. Similarly very aggressive reagents, reactions that form more than one product or are difficult to remove will be less suitable than those which require mild conditions, have few side reactions and are less toxic (See Chapter 5 Cocaine metabolites).

Pre--column derivatisation is carried out mainly in an off-line mode. and its advantages are:

1. No restriction on the reaction kinetics, provided the reaction is completed within a reasonable length of time.

2. There is a free choice in varying the conditions to optimise the reaction time and yield.

3. The solvent in which the reaction takes place can be independent of the mobile phase, making it easy to use aggressive conditions.

4. Chromatographic properties can be optimised.

The disadvantages are:

1. The formation of side products and artefacts, which may interfere with the chromatographic analysis or in the reproducibility of the derivatisation procedure, may occur.

2. Each sample needs individual handling, making it time consuming, and more prone to error.

3. Often an internal standard is needed in case the derivatisation reaction is incomplete.

4. Target compounds may be more alike after derivatisation.

Pre-column derivatisation can also be accompanied by liquid-liquid extraction or another pre-chromatographic separation step. Excess reagent is sometimes removed by reacting it with an excess of another compound, however the other compound or the product formed must not interfere with the analysis.

With recent trends in automation a large number of companies e.g Spectra Physics and Applied Chromatographic Systems, market systems which can carry out 'on-line' pre-column derivatisation. Derivatisation takes place in the injection needle and the sample loop and mixing is controlled by moving liquids back and forwards. Examples include amino acids in biological and pharmaceutical samples (Schuster, 1988) and derivatization of fatty acids with bromomethoxycoumarin (Wolf and Krof, 1990).

1.2.2. Post-chromatographic derivatization

Post-chromatographic or post-column derivatisation is done mainly to improve sensitivity and selectivity and is carried out mainly in an 'on-line' mode as the sample elutes from the column, in a reactor positioned between the column and the detector. This reactor consists of :- a pump to add the reagent, a mixing device where the reagent and the column effluent are mixed, and a coil. The dimensions of this coil depends upon the hold-up time required for the reaction.

The advantages of post-column derivatisation are:-

1. The analytes are in their original form and this allows the adoption of published chromatographic procedures.
2. Unlike pre-column derivatisation, the detailed knowledge of the reaction product is not essential, nor is the product required to be stable, the only requirement is reproducibility.
3. It is performed on-line, requiring very little time and less consumption of reagents so that a large number of samples can be processed within a short time.
4. Signal improvement can be achieved by multiderivatisation of the analyte.

The disadvantages are:

1. Restricted freedom in the choice of reaction conditions.
2. Extra-column band broadening caused by the reactor, resulting in a loss of chromatographic resolution.
3. The need to add reagents to column effluent requires an extra pump, thus increasing the cost of the instrumentation.
4. The excess reagent, or reagent degradation products may interfere with the signal of the reaction products and therefore their removal is important. This may involve modification to instrumentation, increasing further the cost of instrumentation.

Although post-column derivatization is generally performed 'on-line', Lingeman and Underberg (1990) have used the term 'off-line' post-chromatographic derivatization to describe an analytical method where the chromatographic procedure is used for sample clean-up, followed by derivatization and then another separation step to separate the resulting

derivatives. In some cases pre-colum and post column derivatization can be used in combination Boppana et al, (1992).

A great deal of work has been done by Frei et al. to make post-column derivatization a useful technique for many diverse analytical problems. A complete discussion of principles and techniques involved is beyond the scope of this thesis. Therefore the reader is referred to a very recent review article on post-column detection by Brinkman et al (1989) and various books Frei and Lawrence (1981), Frei & Zach, (1988) and, Zach & Frei, (1989). However a brief overview of their work with selected examples is given to illustrate how post-column derivatization can improve the detection of a wide variety of analytes.

The above authors have categorised post-column reactors into :- open tubular reactors (OTR), packed bed reactors (PBR), membrane reactors, photochemical reactors and segmented stream reactors, depending upon the type of reactor attached between the column and the detector. Open tubular reactors consist of a straight or helically coiled or knitted piece of PTFE, glass, or stainless steel tubing, the dimensions of which are primarily determined by the hold-up time required for the selected reaction. Due to problems with axial dispersion, open tubular reactors are used only for short reaction times of around 1 minute. Examples include the reaction of amino-acids with o-phthaldehyde (OPA) (Cunico and Schalabach, 1983). Using knitted reactors Engelhardt and Nue (1982) have demonstrated the use of open tubular reactors for reaction times as long as 4 minutes without excessive band spreading.

Packed bed reactors consist of short stainless steel columns packed with inert glass beads (10-15 μm). Their mixing characteristics and minimal band spreading in comparison with open tubular reactors makes them ideal for long reaction times i.e., 4 minutes. Examples include the detection of amino-acids with OPA (Deeldar et al 1978) and chloranilines with Fluram (Scholten et al 1981). Packed bed reactors can also be packed with 'active materials' like ion exchange resins or immobilized enzymes, thus eliminating the need for using a pump and mixing device to add reagent to the column effluent. Examples include, the catalytic hydrolysis of N-methylcarbamates, where the column effluent is passed through a PBR

packed with Aminex A-28 tetralkylammonium anion exchange resin. The liberated methylamine is then reacted with OPA and detected in sub nanogram quantities fluorometrically (Nondek et al 1983). Another example is the determination of acetylcholine (Ach) and choline in tissue homogenates (Eva et al 1984 & Meek and Eva, 1984), where the column eluent is passed through a PBR packed with an anion exchange resin on which acetylcholinesterase and choline oxidase are immobilised. Here Ach is first hydrolysed to choline, which is then oxidised to hydrogen peroxide and detected amperometrically. The minimum detectable amount ranged from 0.3 (in brain) to 5.0 pico moles. According to Brinkman et al 1989 the results are comparable to GC-MS but the technique is less expensive and time consuming.

Membrane reactors also avoid the use of reagent pumps and mixing tees, and thus give very little band spreading and base line noise which may result from mixing of the column eluent with the reagent stream. In one configuration developed by Dionex, the mobile phase passes through a semi-permeable hollow fibre which is immersed in a solution of post-column reagent under pressure. As a result of osmotic diffusion the reagent enters the hollow fibre and reacts with the analyte. (Wienberger and Femia, 1988). Hollow fibres have also been used for enhanced detection of nitrophenols (with ammonium hydroxide), detection of amines (benzylamine with Fluram) and detection of amino-acids with ninhydrin (Davis & Peterson ,1985).

In a photochemical reactor the analyte, present in the HPLC eluent, is irradiated with a medium or high power UV source in a quartz or PTFE reaction capillary (transmissive for > 200nm). Reactions times are usually 10-60 seconds. Photochemical reactions are generally hard to predict and control because of the formation of a large number of side products (de Ruiter 1989). Methotrexate and its metabolites are converted into highly fluorescent products with a detection limit of 0.4 ng ml^{-1} (Salamoun et al 1987). Ciprofloxacin and its metabolites, after elution were heated in a stainless steel capillary to achieve hydrolysis and then irradiated in a PTFE capillary and detected by fluorescence, achieving a minimum detectable limit of 2.5 - 25 pg (Scholl et al ,1987).

In segmented stream reactors the column effluent is segmented by air or an immiscible liquid. They are widely used in continous-flow analysers and since 1969 as post-column detection systems (Ertingshausen et al 1969). Segmentation is an effective way to reduce the band spreading of a sample or analyte. Reactions which require a long time i.e 23 minutes (Werkenhoven-Goewie et al, 1980) can be carried out with a minimum of band spreading, as segmentation reduces the axial diffusion of the sample zones. Solvent segmentation is preferred to air segmentation because liquid segmentation is not greatly influenced by small variations in pressure, temperature or flow, because of the lower compressibility of liquids. Solvent segmented reactors have found wide-spread use as extraction detectors (See Chapter 3 of this thesis for more details).

Segmented stream reactors require the use of a desegmenter {a debubbler or a phase separator} and this has been found to be a major cause of band spreading (Lawrence et al 1979 a). An alternative approach to physical desegmentation has been to measure only the segments of interest (van der Wal, 1983).

Solvent segmented flow reactors can be used for a variety of applications, for example post-column ion-pair extraction (Lawrence et al 1979 a), flow-injection analysis (Karlberg and Thelander, 1978), LC-MS coupling (Apffel et al, 1984), LC-ECD coupling (Marris et al, 1986) LC-FTIR (Shah and Taylor, 1989) and on-line phase-transfer catalysis (Halvax et al, 1992).

1.3 Ion-pair extraction

For ionisable compounds which lack active sites for derivatization, such as tertiary and quaternary amines and some sulphonates, ion-pair formation with fluorescent or UV active counter-ions, with simultaneous liquid-liquid extraction is a useful alternative technique for increasing the sensitivity and selectivity. In a post-column ion-pair extraction system after HPLC separation, the eluent is mixed with an aqueous solution of the reagent and an immiscible organic solvent. After reaction and extraction in an extraction coil, the aqueous phase containing the excess reagent is removed. The clean organic phase containing the ion-pairs is monitored by the detector. For a successful operation of an ion-pair extraction

detector a phase separator is needed. The most commonly used ion-pairing agents are 9,10 dimethoxyanthracene sulphonate (Westerlund and Borg, 1973) for amines and other basic compounds while acridine (Smedes et al, 1982) is used for sulphonates and other acidic compounds (for more details of ion-pair extraction detectors, see chapter 4 of this thesis).

Ion-pairs are defined as Coulombic association species formed between two ions of opposite charges (Tomlinson et al, 1978). The ion-pairs are held together by 'solvophobic effects' which permit ion association to occur. The major property of ion-pairs is an ability to move from an aqueous environment (high polarity) to organic solvents (low polarity). With ion-pair association the overall transfer between phases is determined by the extent of ion-pair formation, the nature of the formed species and the properties of the extracting phases. A detailed discussion on ion-pair solvent extraction is found in Chapter 7 of this thesis.

Ion-pairing can be used in analytical chemistry for (a) sample preparation, i.e extraction of highly hydrophilic ions from biological matrices (See Chapter 6, where Ruyter et al (1980) used ion-pairing for sample preparation of quaternary ammonium ions),(b) ion-pairing chromatography, generally using UV transparent reagents and (c) to improve detectability, using counter-ions which are UV active or fluorescent.

The extraction of un-ionised substances or weakly acidic or basic substances is relatively easy and can be done either by changing the pH or the organic phase. On the other hand, ionisable substances present in complex samples such as blood or urine etc are highly hydrophilic, strongly polar and often quite ionisable at all pH values, thus making their extraction a problem. They can however be extracted as complexes with other ions of opposite charges i.e as ion-pairs. Extraction of complexes as ion-pairs has been reported in the literature as early as 1931 (Schill, 1974) and since then has been used for the extraction of a large variety of cations and anions.

Ion-pair chromatography is used for the separation of highly hydrophilic analytes, by ion-pairing with a hydrophobic counter-ion the analytes are retained longer on the column away from the interfering peaks of the sample matrix. The selectivity in the system can be varied by manipulation of ion-pair effects i.e by changing the nature and concentration of counter-ion,

changing pH or by changing the nature or proportion of organic modifier. Ion-pair chromatography has been used in this thesis for the chromatographic separation of quaternary ammonium compounds (see chapter 7).

1.4 Chromatography in post-column ion-pair extraction detectors

1.4.1 Reversed phase liquid chromatography

More than 80% of all separations in HPLC are performed using silica based reversed phase systems (RP-HPLC), where a non-polar stationary phase is used with a polar mobile phase. This is because they offer higher column stabilities, lower equilibration times and higher retention reproducibilities than normal phase or ion-exchange systems. As discussed earlier in Section 1.2.2, one of the major requirements for a post-column reaction detection system is that the chromatographic conditions should be compatible with the derivatization conditions. It is therefore appropriate to discuss retention in reversed phase systems in this section.

The separation mechanism in RP-HPLC systems is basically a combination of solvophobic and silanophilic interactions, which means that on ordinary alkyl bonded silica columns the neutral analytes are retained mainly on account of their hydrophobicity. The silanol groups of the silica surface are very reactive and form the basis on which all bonded base chromatography is based. Some of these silanols remain unreacted during the bonding procedure (see Chapter 4) and are responsible for retention of ionisable analytes such as amines, which then elute as badly tailing peaks. However these columns can be deactivated either by attaching bulky ligands (Kirkland et al 1989), by polymeric coating of silica surface (Schomberg et al, 1983) or by attaching a competing nucleophile close to the silanol groups e.g Suplex™ pKb-100 column (Ascah and Feibush, 1990).

Post-column ion-pair extraction detectors require the use of mainly aqueous eluents, as large amounts of organic modifiers increase the background signal i.e., the free ionic form of the counter ion becomes dissolved in the organic modifier, which in turn is miscible with the water immiscible organic solvent leading to higher background signal and greater signal-to-

noise ratio (Gfeller et al., 1979). Therefore stationary phases with a high carbon loading are not suitable for post-column ion-pair extraction detection systems as they require mobile phases with a high organic content. Polar stationary phases e.g cyano, amino, diol are preferred over more non polar ones e.g ODS 2 as they give less retention, and can be used with predominantly aqueous mobile phases.

For the chromatography of bases the addition of mobile phase additives such as TEA (triethylamine) will also lead to a higher background signal as the positively charged TEA forms an ion-pair with the negatively charged counter-ion, leading to a loss of sensitivity. Base-deactivated columns are therefore ideal for post-column systems as they do not require such mobile phase additives.

1.4.2. Narrow bore columns

Narrow bore columns are defined as "conventionally packed columns with reduced internal diameter from 1-3 mm" (Mallet and Law, 1991). These columns offer the advantages of lower mobile phase cost and an increase in mass sensitivity.

To gain the advantages of mass sensitivity the extra column dispersion should be as small as possible as demonstrated by several authors (Mallet and Law 1991, Dolan and Lommen, 1990, Gill and Law, 1986). This requires having a minimum dead volume and a modification of the existing conventional HPLC equipment especially for gradient elution. Most manufacturers supply pump heads, injection valves, and flow cells which can be used for microbore HPLC. Mallet and Law have demonstrated that up to 50 µl can be injected on a microbore column using peak compression. This involves dissolving the sample in a solvent which is weaker than the mobile phase. They have also shown that conventional HPLC detectors with normal flow cells can be used with microbore HPLC.

1.4.3. Gradient elution in HPLC

In gradient elution the composition of the mobile phase changes during the separation. Usually a binary solvent is used, with a strong solvent increasing in concentration during the gradient. Sometimes a ternary mobile phase is also used. Gradient elution is ideal for

separating samples which cannot be handled by ordinary isocratic methods because of a wide k' range. Gradients can either be linear, step, convex or concave and gradient elutions systems can be designed on either high pressure or low pressure mixing of solvents.

1.High pressure gradient systems

The output from two high pressure pumps is programmed into a mixing chamber before flowing into the column. Since the output of each pump can be separately controlled it is possible to generate any type of gradient. The disadvantages of high pressure systems are: a) they are expensive as two high pressure pumps and a gradient controller are required. b) reciprocating piston pumps operate with low precision at low flow rates e.g at 0.1 ml/min unless they are specially modified for microbore columns. c) generally high pressure systems are limited to only binary solvents.

2. Low pressure gradient systems

Gradients are formed by mixing two or more solvents at atmospheric pressure, which are then pumped to the column via a single high pressure pump. A series of solvents of increasing solvent strength are used. Separation is started with a weak solvent and a gradient is produced by sequentially opening valves leading to reservoirs containing mobile phase of increasing solvent strength The concentration of these solvents is selected by controlling the time that the valves to various solvents are opened or closed using a microprocessor controlled system. After that the generated solvent mixture is fed to a single high pressure pump for pressurization. Low internal volume reciprocating piston pumps are used for low pressure mixing to minimise delay time before the new solvent composition reaches the column during a gradient. Using a flow rate of 1 ml/min the lag time is often around 5 min. Despite this disadvantage, low pressure gradient systems are preferred as they offer greater versatility than high pressure systems and are capable of handling a series of solvents of increasing solvent strength. As one pump is involved they are less expensive than high pressure systems. Due to these advantages low pressure gradient systems are widely used.

3. Gradient elution in narrow bore HPLC

Most manufacturers market systems for gradient elution for narrow bore to microbore columns. These consist of, either syringe pumps e.g Isco or LKB (SMART SYSTEM ™) or reciprocating piston pumps e.g Waters (Schwartz et al., 1983). These systems are high pressure gradient systems (HPGS). To improve the accuracy at low flow rates of 1- 10 $\mu l ml^{-1}$ an ACCURATE™ micromixer and splitter has recently been introduced by LC Packings (Chervet et al., 1991). According to these authors, ACCURATE™ can produce highly reproducible flow rates of 1-10μl^{-1} under isocratic or gradient conditions and compensates for any changes in viscosities during gradient elution. It offers the advantages of excellent reproducibilities, low dead volume, virtually no dispersion, low base line noise, ease of use, and inexpensive upgrading of pumps into micropumps.

Low pressure gradient systems are difficult to use with narrow bore or microbore columns, because of the delay time as shown in Figure 1.1 (a), which in the case of a conventional system for a flow rate of 1 ml/min is about 5 min. However, if a large volume mixer is added or the flow rate is reduced to 0.2 to 0.4 ml min^{-1}, this causes gradient dispersion resulting in gradient rounding as shown in Fig 1.1. (b). In Figure (c) the gradient is erratic, while in (d) the gradient is not linear. Equipment that produces these type of gradients (b-d) are less suitable for reproducible and precise analysis by gradient elution (Snyder et al., 1988).

1.5. Aims of work

1 Evaluation of a dual channel phase separator designed by Dr. T.M. Jefferies and made by Scientific Systems Inc (SSI) U.S.A, and design of a post-column ion-pair extraction system.

2. Examination of various stationary phases for use in post-column ion-pair extraction detector systems and the use of the post-column ion-pair extraction system to improve the sensitivity and/or selectivity of selected drugs of abuse, compared to 205 nm (Badiru, 1989).

3 Examination of pre-column derivatisation techniques for improving the chromatography and detectability of highly hydrophilic molecules such as benzoylecgonine and ecgonine methyl ester, and the use of SPE for their sample preparation from urine.

4. To demonstrate the use of post-column ion-pair extraction system for enhanced sensitivity and selectivity of some quaternary ammonium compounds

5. Evaluation of some fluorogenic dyes for use as potential post-column ion-pairing agents, in terms of fluorescence sensitivity and extraction constants.

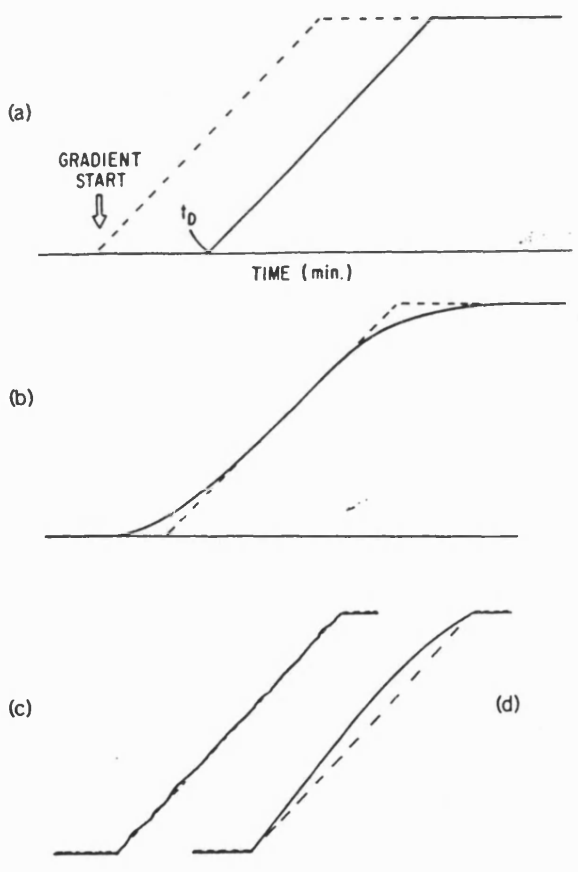


Fig 1.1 Gradient profile for different HPLC equipment. Standard conditions ; gradient time (t_G)=20 min, F=2ml/min, t_D= dwell time/flow rate, Linear gradient from 0-100%. Theoretical gradient - - - - - - - , experimental gradient ----------.

2 EXPERIMENTAL

2.1 Materials

All materials were used as received unless otherwise stated.

HPLC grade methanol (MeOH), acetonitrile (ACN), isopropanol (IPA), tetrahydrofuran (THF), acetone, chloroform ($CHCl_3$), dichloromethane (DCM), dichloroethane (DCE), 9,10 dimethoxyanthracene sulphonate sodium salt (DAS), tetrabutyl ammonium bromide (TBA), were obtained from Fisons, Loughborough. U.K.

Potassium dihydrogen orthophosphate (KH_2PO_4), disodium hydrogen orthophosphate (Na_2HPO_4), were analytical reagent grade, orthophosphoric acid SG 1.69 (H_3PO_4), anhydrous magnesium sulphate ($MgSO_4$), hydrochloric acid SG 1.18 (HCl), sulphuric acid 98% (H_2SO_4) were specified laboratory reagent grade (SLR), potassium carbonate (K_2CO_3) , technical grade were also from FSA, Loughborough.

All chemicals used for synthesis or otherwise were of 96-99% purity and were obtained from Aldrich, Gillingham, Dorset, except neostigmine, pyridostigmine, and edrophonium which were obtained from Sigma Chemical Company (U.K) Poole, Dorset.

Biphenyl, quinine sulphate and quinine bisulphate were GPR (general purpose reagent) from BDH, Poole. N-methyl tertiary-butyldimethylsilyl (MTBSTFA) was obtained from Pierce Warriner, Cheshire. Naphthalene -2 sulphonic acid was obtained from Eastman Kodak, Rochester, N.Y. Water was single distilled from an all glass still and filtered through a 0.45 µm cellulose nitrate membrane.

Solvents used for derivatization reactions (section 5) were stored over activated Size 4A molecular sieves and were obtained from Aldrich, Gillingham, Dorset. Reacti-vials for derivatization reactions were obtained from Supelchem U.K Ltd, Essex. For derivatization the vials were heated in a hot air oven at specified temperatures.

The drugs of abuse classified as Group A and B on account of their chromatographic behaviour (Badiru, 1988) were obtained from the Central Research Establishment, Forensic Science Service, Home Office, Aldermaston. Cocaine hydrochloride was obtained from the dispensary of School of Pharmacy and Pharmacology, University of Bath, Bath U.K.

Urine and plasma samples were obtained from drug free individuals, and immediately after collection were divided into 1.0 ml portions and stored at -4^0 C. The samples were thawed prior to use. Commercial urine samples were obtained from Bio-Rad laboratories Ltd., Hemel Hemstead.,

2.1.2 HPLC columns and packing materials

The properties of HPLC packing materials used in this study are given in Table 2.1 Hypersil BDS, Nucleosil SA and Spherisorb SCX (150 X 2.1 mm i.d) columns were packed by Capital HPLC (Edinburgh). Nucleosil SA (50 X 4.6 mm i.d) column was packed in-house. Spheri-5-cyano Brownlee cartridge column (100 X 2.1 mm i.d) was obtained from Anachem, Beds, U.K. Methyl-cyano (100 X 2.1 mm i.d) column was a gift from Phase Separations, Deeside, Clwyd. SGE columns (100 X 2.1 mm i.d) were a gift from Scientific Glass Engineering (UK) Ltd, Milton Keynes. PLRP-S (150 X 4.6 mm i.d and 2.1 mm i.d) columns were obtained from Polymer Laboratories, Shropshire, U.K. Supelco pKb-100 columns (250 X 2.1mm i.d) columns were a gift from Supelco Inc U.S.A.

2.1.3 Equipment

Most of the analytical studies were carried out using a Scientific Systems Inc., State College, PA, USA, ternary gradient system (model GS 402) consisting of SSI model 220 B single piston pump fitted with a pulse dampener, model 231 gradient controller, a Rheodyne model 7125 manual injection valve fitted with a 10, 20 or 50 µl loop, or an SSI

Table 2.1 Properties of HPLC packing material used

Column	type	%C w/w	S.A	End capping	Particle size μm	Pore size A^0
Brownlee	Spheri- -5 cyano	3.5	220	-	5	80
Phase Sep	methyl-cyano-polymer	10	220	polymer coating	5	80
Hypersil CPS	cyano-propyl	3.6-4.4	170	-	5	120
SGE Cyano	cyano-propyl	3.5	220	-	5	80
SGE C_8		12	220	fully	5	80
SGE phenyl	- phenyl	3	220	partially	5	80
Nucleosil C_{18}	ODS	14		fully	5	*
Hypersil BDS	Base deactivated C_{18}	7	170	fully	5	120
Supelco pKb 100	proprietary	*	*	*	5	*
Nucleosil SA	Cation exchange	*	*	*	5	*
Spherisorb SCX	strong cation exchange	*	*	*	5	*
PLRP-S	PSDVB	*	415	*		75

% C w/w percentage carbon loading

S.A = surface area m^2/gm

A^0 = Angstrom

* information not available

XL injector with an internal loop (0.2, 1.0 and 10 µl) system. The samples were injected using either a 50 µl or 100 µl fixed needle syringe from SGE.

The HPLC column was enclosed in an SSI Model 505 column oven. For U.V studies model 500 variable wavelength detector from SSI or Spectromonitor 3100 (variable wavelength detector) from LDC, were used. For post-column fluorescence detection most of the work was carried out using a Perkin Elmer model PE 204 S spectrofluoromonitor fitted with a 150 µl flow cell, with a 150 Xenon power supply and attached to a Servogor chart recorder or HP 3390 integrator or Spectra Physics model SP4270 integrator. However at various stages a filter Fluoromonitor with a 30 µl flow cell (LDC) and an HPLC fluorescence detector Model 820 FP with a 16 µl flow cell from JASCO, Ciba Corning Analytical, Essex , U.K. were used.

Fluorescence spectra were recorded on a Shimadzu RF 540 spectofluorometer. Off-line fluorescence studies for the determination of extraction constants were performed on a PE 204 S in 'ratio mode'.

The post-column system was assembled from two model 350 single piston (flow rate 0.1-1.5 ml min^{-1}) pumps attached to either a Model 210 Guardian pulse dampener (SSI),or a LO-PULSE dampener (SSI), additional pulse dampening was provided by 100 X 4.6 mm i.d columns packed with 10 µm Partisil silica (aqueous phase) and CPS Hypersil (organic phase). The addition of the aqueous reagent to the HPLC eluent and the segmentation of the aqueous phase with the organic phase was achieved using Tee connectors, 1/16 X 0.015 inch (SSI). An Uptight precolumn, 20 X 2 mm i.d., packed with 75 µm glass Ballotini beads was used as a mixing chamber. The extraction coil was made either of 0.8 mm i.d or 1.0 mm i.d PTFE or stainless steel of various lengths and coil diameter. The phase separator (designed by Dr T. M. Jefferies) was made by SSI and the exit channels for the aqueous and organic waste were controlled by MCV - 50 micro needle valves donated by SGE Milton Keynes.

Solid phase extraction cartridges 'Bond Elut' CBA , SCX , Certify (130 and 300 mg) were purchased or were donated by Varian (Jones Chromatography, Hengoed, UK.). Narc 2 was a gift from J.T. Baker UK Middx, Clean Screen DAU (World Wide Monitoring) and MP-3 (Interaction Chemicals) were a gift from Technicol (Stockport). Sample preparation using these solid phase extraction cartridges was done using Vac Elut-24 purchased from Jones Chromatography, Hengoed, U.K.

2.1.4 Preparation of mobile phase and reagents

Phosphate buffers were prepared by mixing 0.01 M aq KH_2PO_4 with 0.01 M H_3PO_4 or 0.01 M KOH to the required pH. The pH of the solution was checked using a Kent EIL 7020 pH Meter, calibrated with aqueous potassium hydrogen phthalate buffer at pH 4.0. The aqueous portion of the buffer was filtered through a 0.45 μm cellulose nitrate filter before addition of organic modifier. All components of the mobile phase were mixed volumetrically. DAS was dissolved in filtered water or filtered buffer. All mobile phases were degassed by sparging with helium or by ultrasonicating for 15 minutes.

2.2 Methods

2.2.1 Operating conditions

For all 2.1 mm i.d columns, whenever possible the flow rate selected was 0.4 ml min^{-1} except for Spheri-5 cyano and methyl cyano where it ranged from 0.5-0.8 ml min^{-1}. For 4.6 mm i.d columns the flow rate was 1.0 ml min^{-1}.

All columns were enclosed in an SSI column oven and held at a stated temperature

2.2.2. Operation of the post-column system and the phase separator (Fig 3.7)

The HPLC column is connected to the post-column system but the phase separator is not attached to the detector. See Fig 3.1

1. Turn on the HPLC pump and after the pressure has stabilised, turn on the reagent pump .

2. Turn on the organic pump.

3. Open the needle valves (2 and 3) of the phase separator fully.

4. Flush the flow cell of the detector with methanol, using a syringe connected to a luer fitting.

5 By the time the detector flow cell is flushed, clean organic flow, free from water droplets should be passing through outlet '4' of the phase separator.

6. Connect outlet '4' of the phase separator to the 'inlet' of the detector flow cell using a zero dead volume fitting.

7. When the organic flow starts flowing though the detector outlet, close the phase separator needle valves (2&3) slowly by turning them clockwise. This is done to increase the flow of the organic phase through outlet '4' and the detector. Measure the output in a measuring cylinder.

8. The separation efficiency will be around 80%, when the needle valves are 50% closed. At this stage the outlet '3' of the phase separator will have plain aqueous phase, and exit '2' will have aqueous phase with small droplets of organic phase.

9. For some detectors it is beneficial to attach a back pressure regulator to the outlet tubing of the detector in order to eliminate the base line disturbances caused by air bubbles. Under such conditions the needle valve needs to be closed to about 75% to achieve the same separation efficiency.

10. Leakage of the aqueous segments into the flow cell may occur due to (a) closing the needle valves too much,(b) blockage in the exit tubing of the needle valves,(c) malfunctioning of the aqueous or organic pumps, or (d) leakage in the tubing connecting the back pressure regulator to the detector. If this occurs, then open the needle valves 2 and 3 fully, disconnect outlet '4' from the detector and repeat the whole procedure from step 4, after taking steps to correct the fault.

2.2.3 Modifications made to the gradient Controller

The modifications to the gradient controller were necessary in order to reduce the delay time, i.e time from the onset of the gradient to the moment it reaches the top of the column, in order to obtain reproducible gradients at low flow rates i.e 0.2 to 0.4 ml min^{-1}. The accuracy tests for linear and step gradients were performed according to the SSI manual.

Mobile Phase A: Methanol

Mobile Phase B: Methanol-0.2 % aqueous acetone (75:25 v/v).

Flow rates as specified In TABLE 2.2,

Detection 0.5 AUFS @ 254 nm.

A small 20 X 2.0 mm i.d column was used as a restrictor between the pump and the detector.

Linear gradient

time	%A	%B	Gradient type
Initial	100	0	*
1.0	100	0	linear
10.0	0	100	linear
20	100	0	linear

Step gradient

Step-wise gradients with compositions of 0 , 2 , 5 , 1 0 , 3 0 , 5 0 , 7 0 9 0 , 9 5 , 9 8 and 100 % B were used. The time interval between each step was 5 minutes so that the solvent composition was stabilised after each step.

The following configurations were used:

Config 1

Model 220 B with standard pump head (1-10 ml min^{-1}) and 200 X 1.8 mm i.d tubing connecting the gradient controller to the pump head (standard connection)

Config 2.

Model 220 B pump with microbore pump head (0.025-5.0ml min^{-1}) and 150 X 1.8 mm i.d tubing connecting the gradient controller to the pump head.

Config 3

Same as Config 2 but with 100 X 0.8 mm i.d. tubing.

Config 4

The injection valve was connected directly to the purge by-passing the inlet filter using a 200 X 0.8 mm i.d tubing. Other conditions as in Config 3.

Results

The results are shown in TABLE 2.2. The delay time was measured from the linear gradient as shown in Figure 2.1. The angle of the step was measured by drawing a tangent to the slope as shown in Fig 2.2 and the angle measured at that point.

For perfect steps, the angle measured should be 90^0. Configuration 4 (125^0) has produced improvements over configuration 3 and 2.

TABLE 2.2

Config	flow rate ml min^{-1}	type	Delay time (min)	type	Angle of step ^{0}
1	1	linear	4	step	115^0
1	0.2	linear	18	step	n.m
2	0.4	linear	6.8	step	130^0
3	0.4	linear	6.4	step	127^0
4	0.4	linear	4.4	step	125^0
4	0.2	linear	8.2	step	132^0

Further improvements in the system could be made by decreasing the volume of the pulse dampener and the purge valve. Changing the software of the pump i.e funkey factors and the volume quotient did not make any noticeable change in performance. As Config 4 gave the shortest delay time and the shortest angle of step, it was therefore used for subsequent gradient work.

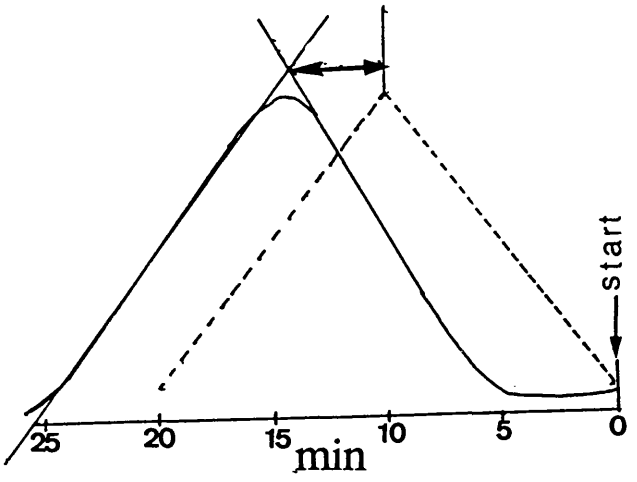


Fig 2.1 Linear gradient Config 4: Delay time, - - - - - - theoretical gradient, ——— experimental gradient

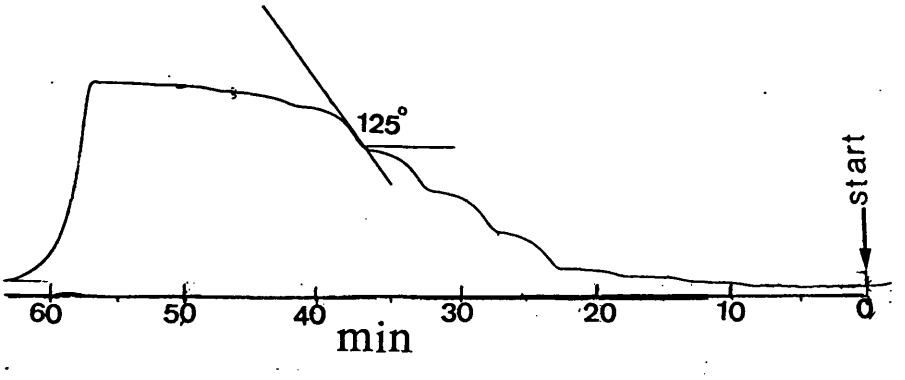


Fig 2.2 Step gradient Config 4: Angle of step.

2.2.4 Solid phase extraction procedure (Varian)

Solutions

A. Phosphate buffer pH 6.0 (1 litre) : Weigh 13.61 gm of KH_2PO_4 (MW 136.09) into a 1.0 L volumetric flask. Dissolve the KH_2PO_4 into 900 ml of water. Adjust the pH to 6.0 (± 0.1) with 1.0 M potassium hydroxide while stirring. Bring the total volume to 1.0 L with distilled water. Store at 5^0C, discard after 30 days.

B. Potassium Hydroxide 1.0 M (100 ml) : Weigh 5.6 g of potassium hydroxide (MW 56.11) into a clean plastic 100 ml volumetric flask. Dissolve the potassium hydroxide with distilled water and bring to volume. Stable at room temperature for 3 months.

C. Acetic acid 1.0 M (100 ml) : Pipette 5.75 ml of glacial acetic acid into a 100 ml volumetric flask approximately half filled with distilled water. Mix, bring to volume with distilled water. Store at room temperature. Discard after 2 months.

D. Hydrochloric acid 0.1 M (250 ml) : Into a 250 ml volumetric flask add 150 ml of distilled water. To this add 2.1 ml of concentrated hydrochloric acid. Bring to volume with distilled water. Stable at room temperature for 3 months.

E. 2%(v/v) Ammonium hydroxide in ethyl acetate (100 ml): Pipette 2 ml of concentrated ammonium hydroxide into a 100 ml reagent bottle containing 98 ml of ethyl acetate. Shake vigorously or sonicate for 5 minutes to mix. Prepare fresh daily.

F. Methylene chloride: isopropyl alcohol (80:20 v/v) with 2% ammonium hydroxide (100 ml): Into a 100 ml reagent bottle add 80 ml of methylene chloride and 20 ml of isopropyl alcohol. Mix. Remove 2.0 ml of this solution and add 2 ml of ammonium hydroxide. Shake vigorously to mix. Transfer to a capped container. Prepare fresh daily.

Procedure for basic drugs

1. Specimen Preparation:

To a large test tube add : 5ml of urine, 2 ml of phosphate buffer pH 6.0 (A). Check the specimen with pH paper. The pH should be between 5.0 and 7.0. If not adjust appropriately.

2. Bond Elut Certify Preparation

Place the column and a stopcock into the Vac Elut, plugging any unused ports. Pass through the column sequentially: 2 ml methanol and 2.0 ml phosphate buffer pH 6.0 (A). Turn off the vacuum as soon as the buffer reaches the top of the column to prevent the column drying out.

3. Specimen application:

Pour the specimen into the column reservoir. Loosen the Vac Elut flow valve to reduce the vacuum. Draw the specimen through the column. It should take at least 2 minutes to pass the sample through the Bond Elut Certify column (~ 5 inches Hg)

4. Column rinse:

Pass through the column, 1.0 ml of acetic acid (C). Dry the column under full vacuum (15 inches Hg) for 5 minutes. Pass through the column 6 ml of methanol. Dry the column under vacuum for another 2 minutes.

5. Elute: Place a rack with labelled tubes into Vac Elut and wipe the tips of the Vac Elut delivery needles. Pass through the column 2ml of 2% ammonium hydroxide in ethyl acetate (E) and collect. Concentrate, derivatize and inject

Procedure for Cocaine and Benzoylecgonine (130 mg cartridges)

Follow steps 1-3 as for basic drugs.

4. Column rinse:

Pass through the column : 6 ml of water, dry under full vacuum (15 inches Hg) for 5 minutes. Pass through the column sequentially : 3.0 ml of 0.1 M HCl (D) and 9 ml of methanol.

5. Elute cocaine and benzoylecgonine:

Place a rack with labelled tubes into the Vac Elut and wipe the tips of the Vac Elut needles. Pass through the column 2ml of methylene chloride:isopropyl-alcohol (80:20 v/v) with 2% ammonium hydroxide.(F) and collect . Concentrate, derivatize and Inject.

3 OPTIMISATION OF POST-COLUMN SYSTEM

3.1 Introduction

As discussed in the introduction (section 1.2.2) a post-column system is a device or a series of devices which are connected between the column and the detector, in order to improve the detectability of the analyte without affecting its chromatography.

A post column system generally consists of a pump to add the reagent to the column effluent, a mixing unit and a reactor which may in some cases have a delay volume of several mls. All these extra devices and the tubings which connect these devices add a significant dead volume to the system, which may seriously affect band spreading. Much attention is therefore required to design a post-column system without significant dead volume so that the sensitivity of the method is not compromised.

The choice of a particular post-column system will depend upon the nature of the analyte and the method required for its detection. Most of the compounds studied in this thesis were tertiary amines or quaternary ammonium compounds and chemical derivatization to enhance UV absorption or impart fluorescence was not possible. However these compounds can readily form ion-pairs with suitable UV-absorbing or fluorescent counter-ions and therefore post-column ion pair extraction detection was the preferred choice.

The design and optimisation of post-column ion-pair extraction detectors is discussed below and other reactors such as tubular reactors or packed bed reactors are not covered in this thesis. For design and optimisation of other reactors the reader is referred to Frei and Zech, 1988 and references therein.

3.2 Extraction detectors

Extraction detectors are segmented stream reactors where the column effluent containing the reagent (added post-column or pumped with the mobile phase prior to the column) is segmented with an immiscible liquid which leads to an extraction coil. The two phases are then separated using a desegmentor or a phase separator and the 'clean' phase of interest

(without contamination from the other phase e.g clean organic phase without water droplets) is supplied to the detector and the analytes are monitored.

Fig 3.1 shows the diagram of an HPLC post-column extraction set-up.

The purpose of segmentation is two fold:

1. Segmentation reduces band spreading by dividing the peak of interest into very small volumes (1-2 µl) after leaving the column, in which form it travels down the extraction coil. Segmentation reduces axial dispersion which is responsible for band spreading.

2. The immiscible organic liquid is chosen to extract the lipophilic ion-pairs or derivatised analytes, leaving the unreacted reagent in the aqueous phase. In this way the reagent does not interfere with the analysis.

For example, dansylation reactions of primary and secondary amines are very slow reactions and even at elevated temperatures for some compounds may require 23 minutes to obtain an acceptable signal. The use of a post-column extraction detector not only reduces the band spreading which will occur in this case because of the holdup time of 23 mins. but also removes the excess reagent which is fluorescent even in its hydrolysed form (Wekenhoven-Goewie et al ,1980).

Another example of post-column extraction detectors is the well established ion-pair formation of tertiary and quaternary ammonium compounds using 9,10 dimethoxyanthracene sulphonic acid sodium salt (DAS) as a fluorescent counter-ion. Although the reaction is instantaneous, DAS is fluorescent and its removal is essential for the detection to be selective. For more details see results and discussion of this chapter and Chapters 4, 5, 6 and 7 of this thesis.

The use of post-column ion-pair extraction detectors not only offers the advantages of enhanced sensitivity but also increased selectivity especially for biological or environmental samples. Most biological samples contain large amounts of very polar substances, which are not extracted into immiscible organic solvents, and thus do not interfere with the analysis (placing less demand on sample clean-up). However, this may cause some problems for those analytes of interest which resemble polar components of the matrix e.g metabolites (for details see Section 5 of this thesis).

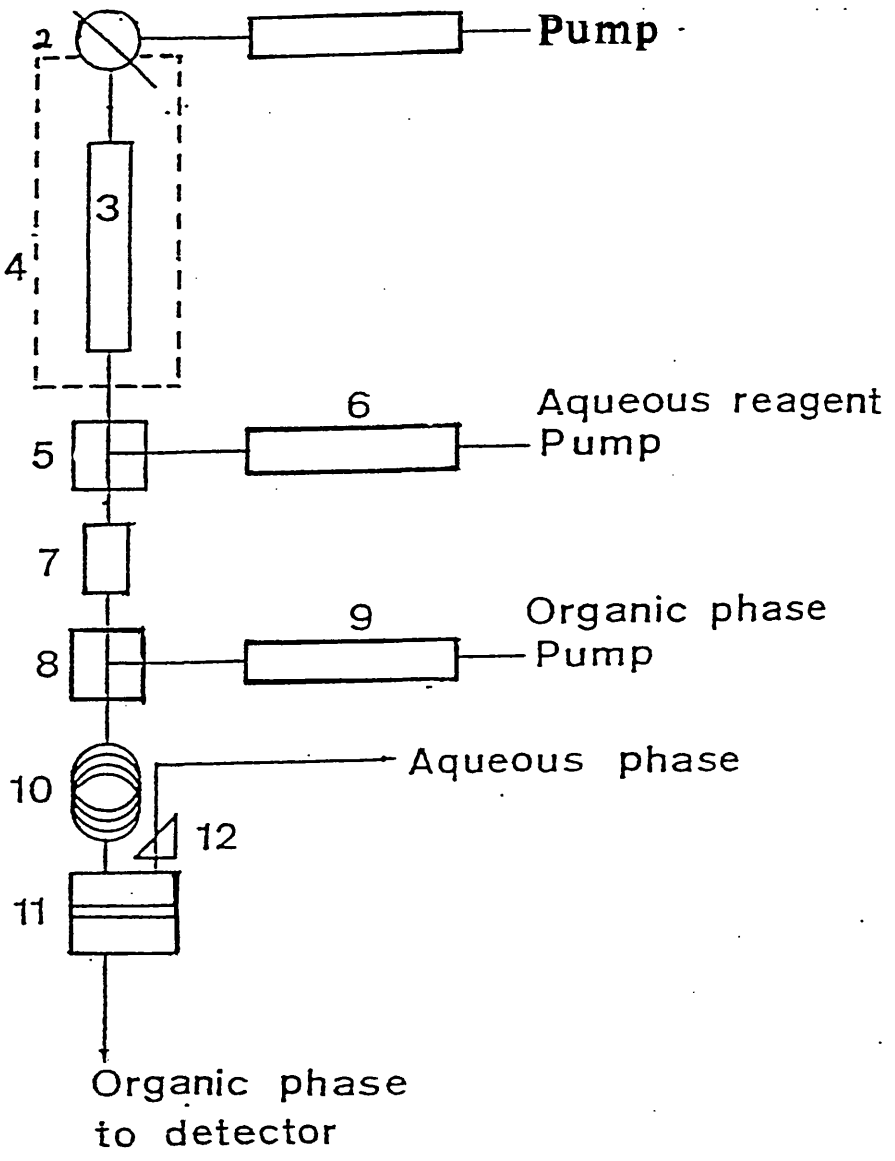


Fig 3.1 HPLC postcolumn system
1,6 & 9 = inline columns, 2= injection valve, 4 = oven, 5 & 8 Tee connectors, 7= mixing column, 10 = extraction column, 11 = phase separator, 12 = needle valves

Since their introduction by Karlberg and Thelander (1978) extraction detectors have found wide spread use without the HPLC column in solvent extraction-flow injection analysis (SE-FIA) units. These units work in the same way as extraction detectors except that there is no column and the sample is injected directly into the aqueous or the organic phase. Dispersion in the FIA system is not a problem as the analysis is very fast, increasing sample through put. In a recent study (Halvax et al.,1990) the FIA method was found to be 5 times faster than an HPLC method which required an analysis time of 7 mins. FIA systems are very suitable for the analysis of compounds where the components of interest are very different from the sample components, so that no separation is required before the analysis. SE-FIA has also found use in the determination of extraction constants (see Section 7) and also for rapid determinations of drug partition coefficients (Kinkel and Tomlinson, 1980). Fig 3.2 shows a typical SE-FIA system.

3.3 Problems with post-column extraction detectors

The major problems associated with post-column ion-pair extraction systems are limitations in the percentage of methanol or acetonitrile that may be used in the mobile phase, interferences from excess reagent and band spreading (Brinkman et al., 1989).

The choice of mobile phase is limited, as increasing the percentage of organic modifier increases the solubility of the counter-ion in the extracting solvent which produces a high background signal. Moreover, mobile phase additives used to prevent tailing of basic compounds, are not permitted as this would also raise the background signal. The use of alcoholic solvents as mobile phase organic modifiers e.g methanol may increase signal to noise ratio especially if chlorinated organic solvents are used as extracting solvents (Reddingius et al., 1981). However addition of alcohols to the extracting solvents can help the extraction (Lawrence et al., 1989 a). The extraction of excess reagent into the organic phase can be overcome by adjustment of pH, or buffer's ionic strength, concentration of counter-ion and selection of the extracting solvent.

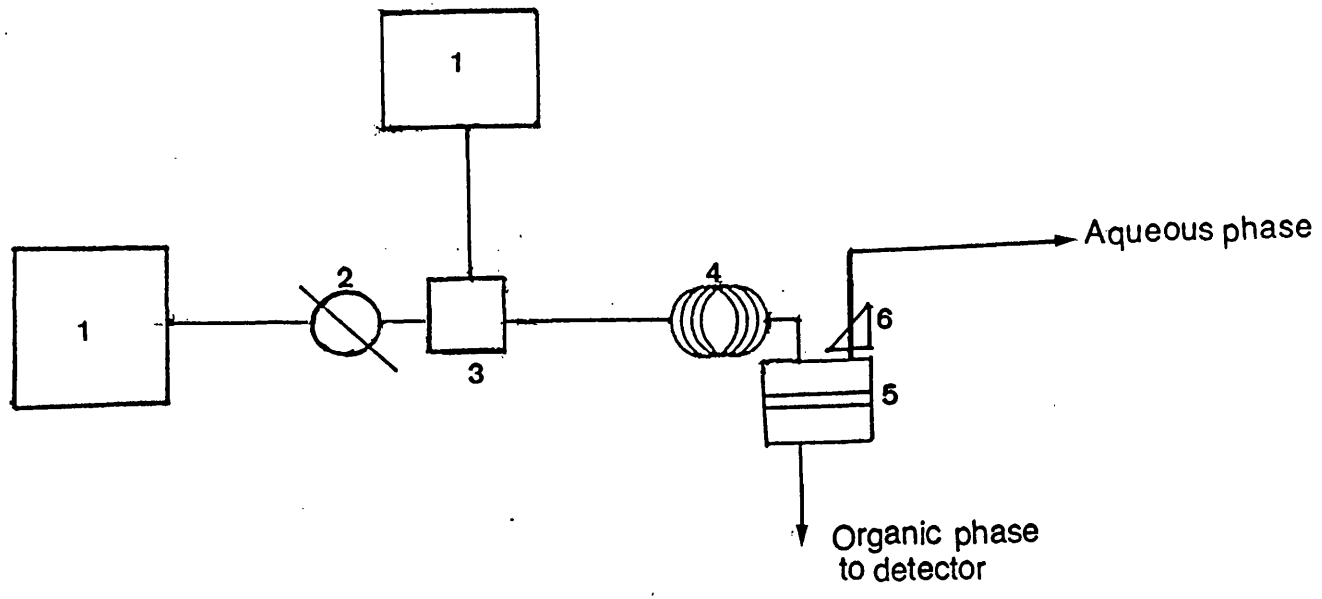


Fig 3.2 Solvent extraction FIA system.
1= aqueous or organic pump, 2= injection valve, 3= tee piece, 4= extraction coil,
5= phase separator, 6= needle valves.

The problem of band spreading can be minimised by optimisation of the hardware components of the post-column system which includes the pumping systems, segmentor 'T', extraction coil and the phase separator. Before discussing the various pieces involved in the post column set-up it is advantageous to discuss the various designs of post-column ion-pair extraction systems reported in the literature.

3.4 Designs of post-column extraction unit reported in literature

The first application of extraction detector principle in combination with HPLC as a post-column unit was developed by Gfeller et al., (1978) and Frei et al., (1979) for the detection of some tertiary amines in pharmaceutical dosage forms. They used the fluorescent counter-ion 9,10 dimethoxy-anthracene sulphonate (Westerlund and Borg, 1973) as an ion-pairing agent. The post-column extraction unit was a Technicon Autoanalyser unit which used peristaltic pumps, and was connected between the column and the detector. Initially a three phase system was used, the aqueous effluent from the column was segmented by air and mixed with the counter-ion in the mixing coil. The organic phase dichloroethane was then added 'on-line' for extraction of lipophilic ion-pairs which were then monitored by a fluorescence detector. Later (Lawrence et al., 1979 a) the air bubble was eliminated as the presence of third phase i.e air at equilibrium decreases the contact between the two liquid phases (Ruzicka and Hansen,1981). The organic solvent was therefore used both as an extracting solvent as well as a segmentor. The band spreading in the two phase system was identical to the three-phase system. Burren et al., (1980) added the counter-ion DAS to the mobile phase prior to the column and eliminated the need for an extra pump. The counter-ion was used not only to improve detectability but also the chromatography of the analytes. Although this approach eliminates the reagent pump and the mixing unit it has some disadvantages e.g where the analytes differ widely in their polarity the addition of a highly hydrophobic counter-ion e.g DAS would retain highly hydrophobic analytes. It also limits the use of gradient elution as increasing the concentration of organic modifier in the mobile phase removes the ion-pairing agent from the column and also increases the solubility of the counter-ion in the

mobile phase giving rise to high background signal as well as long equilibration times. Moreover the chromatography of analytes cannot be checked independently without the use of the post-column set-up for trouble shooting purposes.

The use of a post-column extraction detector has also been demonstrated in normal-phase chromatography (Lawrence et al., 1979 b).

Most of the ion-pair extraction detectors designed today are two-phase systems. Operated either in a two-pump mode (counter-ion added to the mobile phase prior to the column) or a three pump mode (counter-ion added post-column). Post-column detectors place considerable demands on the pumps, mixing devices, phase segmentors and phase separators as the success of the system depends on reducing band spreading in order to achieve maximum sensitivity.

3.5 Components of extraction detectors

a Pumps

Reddingius et al., (1981) observed that designs based on Auto Analysers using peristaltic pumps contributed significantly to the base line noise. A syringe pump was therefore used to supply the organic solvent into the post-column system. Another disadvantage of peristaltic pumps is that rigorous control of the flow rate is not possible because of deterioration of the acidflex tubing used for pumping organic solvents. Sometimes particles from inside the tubing were detached into the system causing blockages (Fossey and Cantwell 1982). The use of HPLC pumps (constant flow or constant pressure pumps) is recommended. Low cost single piston HPLC pumps require the addition of pulse dampeners to minimise pulsations (Pickering, 1988). Syringe pumps are much better, especially at low flow rates, but low cost pumps are not available .

b. Mixing Units

The band spreading that occurs in the mixing units, especially at very low flow rates is a combination of the dilution effects and the dead volume of the mixing unit itself (Scholten et al., 1981). It is important that the streams are homogeneously mixed before they enter the reactor unit. Various geometries of mixing Tees have been suggested e.g. 30°, 60°, 90°, 120°

(the angles refer to the angles between various branches of the tee piece) (Frei and Lawrence, 1981). The 30° version was found to be very efficient for mixing streams which vary widely in their viscosities and flow rates because of the increased turbulence which is produced in the mixing area (Frei et al., 1977). With solutions which are similar (i.e., in terms of viscosity and flow rates) very little difference was observed between the designs (Scholten et al., 1981). Apart from the Tee piece design various other mixing units have appeared in the literature e.g a 25µl rotating device (Kobayashie and Imai 1980), cyclone mixers with a 10 nl volume (Engelhardt et al., 1990). Another mixing device which has been suggested is a short guard column packed with glass beads to give good radial mixing (Frei and Lawrence, 1981).

c. Segmentor

The function of the segmentor is to create regular patterns of aqueous and organic segments which will equilibrate during their passage through the mixing coil. To obtain reproducible results the ratio between equilibrated areas must remain constant (Ruzicka and Hansen 1981) i.e., the segment size must remain constant. Karlberg and Thelander (1978) in the first paper which described the use of extraction detectors, used a modified A8T connector where the organic phase is led into a platinum capillary and the aqueous phase into a glass capillary. A Teflon tube was adjusted into the outlet of the 'T' connector and held in place by an elastic sleeve. This arrangement enabled the authors to control the distance between the orifices of the platinum capillary and the Teflon tube thus controlling the size of segments.

Cantwell and Sweileh (1985) studied the origin of segmentation in the segmentor and found that hydrodynamic force, interfacial phenomena and gravity all play a role in segment formation and that small segments are generally produced at higher flow rates. Motomizu and Oshima (1987) designed and tested various configurations of 'T' segmentors using SE-FIA for ion pair extraction detection. Using peak heights and peak width at half height they found that 'T' segmentors in which the aqueous stream flows straight through and the organic flow is at right angles was found to be the most efficient.

Quinn (1989) examined a conventional 'T' segmentor and a spin chamber (Lee micromixer) with an internal volume of 10 µl for the ion-pair extraction detection of physostigmine with DPS. It was found that the micromixer gave a 7% increase in the extraction of physostigmine compared with the 'T' segmentor but the band broadening in both cases was similar. The effect of using a micro mixer (for mixing organic and aqueous layers) on segmentation was not examined. The micromixer probably disperses the phases as small droplets into each other thereby increasing the contact angle between the phases.

d. Extraction coils

The extraction of lipophilic ion-pairs from aqueous to organic liquid takes place in an extraction coil. The efficiency of extraction can be increased by increasing the length of contact between the two phases, and the simplest way of achieving this is by increasing the length of the extraction coil. This may, however, increase band spreading slightly. The theoretical aspects of extraction in segmented flow have been studied by Nord et al., (1987) and Lucy and Cantwell (1989 a). They observed that in straight tubes, in segmented streams a 'toroidal' circulation pattern is observed i.e., the fluid in the centre appears to be moving forward, while that which is near the sides appears to move backwards because of drag and near the end of segments the flow changes rapidly from axial in direction to radial. In coiled tubes centrifugal forces act strongly on the fastest moving regions of the flow i.e. at the tube centre. As a result of this the fast moving liquid is thrown from the centre and is replaced by recirculating fluid which flows tangentially along the walls. This secondary flow augments the 'toroidal' circulation and increases the rate of extraction. Another important character of segmented flow is that solvents which have a greater affinity for the walls form a thin 'wetting film' on the wall e.g organic solvents such as chloroform have a high affinity for hydrophobic materials like Teflon and form a thin film inside the Teflon tube, whereas aqueous solvents which have a high affinity for hydrophilic materials like glass or stainless steel will form a thin film in a glass or stainless steel tube.

Werkenhoven-Goewie et al (1980) examined stainless steel, teflon and glass coils for band spreading using dansylation reactions of amines. For long reaction times of 2-20 minutes

the band spreading in all the three materials was the same. However the use of PTFE was not recommended because of leakage of aqueous segments into the phase separator and also because of memory effects, the solvents which wet the tube contain a relatively high concentration of extracted solute which would be present at the tubing inner surface (Shelly et al. 1982).

The efficiency of extraction not only depends upon the length of the extraction coil but also on the size of segments and whether the tubes are straight or coiled. Lucy and Cantwell (1989 a, b) summarised their results as: extraction increases (a) by decreasing the tube diameter (as this increases the interfacial area to volume ratio) also found by Motomizu and Oshima 1987, (b) decreasing the segment size, (c) tightening the coiling of the tubing (this effect is greater for long segments than for short segments) and (d) decreasing the linear velocity (at the expense of band spreading) or (e) increasing the length of extraction coil.

Fossey and Cantwell (1982) using caffeine as a model found that the peak width at half height increased up to about 20% as coil length was increased up to an equilibrium point. For coil lengths near the equilibrium point the peak widths became independent of coil length.

In open tubular reactors peak dispersion is reduced by geometrical modifications of the tube by coiling, knitting or stitching (Engelhardt, 1988). Tightening the coil also lowers the band dispersion. However in segmented streams, tight coiling of PTFE tubing with a small internal diameter tubing causes an increase in band spreading because of the merging of segments due to secondary mixing effects (Werkenhoven-Goewie et al 1980).

Quinn (1989) compared crocheted, knitted and coiled PTFE tubing for band spreading and extraction efficiency and found that the crocheted form gave higher extraction but band spreading was also greater. The coiled and knitted forms gave the same extraction efficiency and band spreading.

e. Phase separator

The phase separator is the most important part of an extraction detector unit because it is required to separate the two phases from each other and deliver a 'clean', water droplet free, stream of organic liquid to the detector. Phase separators have an internal volume varying

from 3µl to 0.28 mls, depending upon the design of the phase separator, which can contribute significantly to band spreading. A balance is therefore needed between efficient phase separation, band spreading and other requirements of the phase separator.

3.6 Requirements of a perfect phase separator

1. Complete separation should be achieved for a maximum range of aqueous and organic solvents.
2. Dispersion should be minimum.
3. Material of construction should be inert, so that it can be used with a wide variety of samples.
4. Be easy to use over prolonged periods of time without much trouble.
5. Should have a separation efficiency close to unity, since peak height is directly proportional to separation efficiency.

The design and development of an efficient phase separator is an active area of interest among many research workers. This section covers the development and evaluation of a phase separator designed by Dr. T.M.Jefferies and manufactured by Scientific System Inc., State College. PA. U.S.A , and optimises a design for a post-column extraction unit which will be used for various analytical problems in this thesis. It is now available as a commercial unit from SSI USA.

3.7 Development of Phase separator

Probably the first phase separators reported in the literature were those designed by Stanton and McDonald (1963) and Carter and Nickless (1970). The first one was a modified 'Y' tube and was designed for liquids lighter than water and is shown in Fig 3.3. Fig 3.4 shows the phase separator of Carter, which was designed for liquids heavier than water. According to the designers of the 'T' shaped separator the dimensions of the phase separator were crucial and the dimensions shown in the figure were the best compromise between clean separation and dead volume which in this case was 0.28 mls. Bergamin et al (1978) reported another

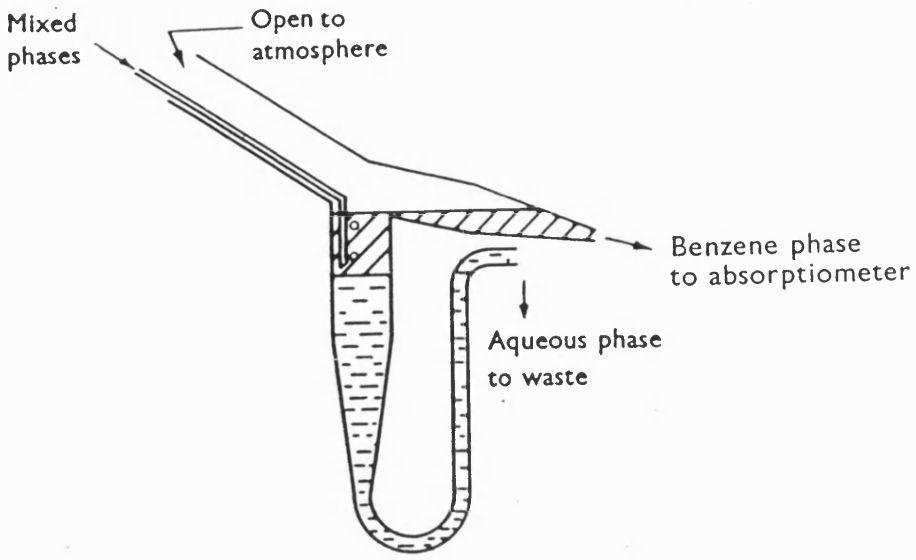


Fig 3.3 Phase separator (Stanton & Mcdonald, 1963)

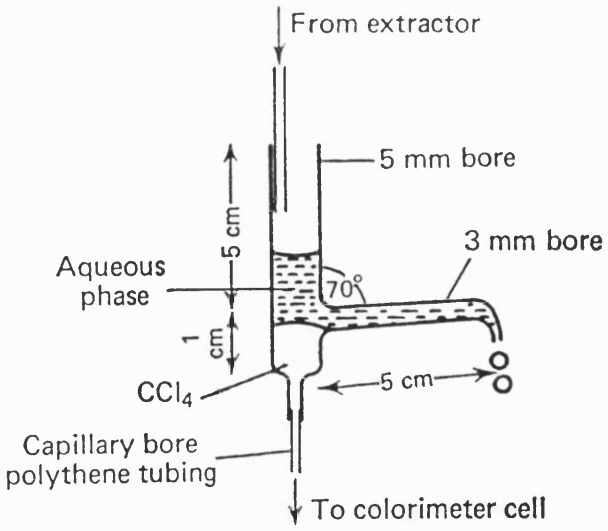


Fig 3.4 Phase separator (Carter & Nickless, 1970)

phase separator which was designed to handle both types of liquids i.e those heavier than water and those which were lighter than water. This phase separator had a dead volume of 0.11 mls. All the three phase separators were designed to work on the basis of the density of phases involved.

In 1978, Karlberg and Thelander published the first of many papers that developed the technique of FIA and described a simple modification of an A4 T-connector made of glass (Technicon, Tarrytown, NY, USA) as a separating device. Teflon fibres in the connector guided the organic phase to the detector and away from the aqueous phase. A similar approach was described by Lawrence et al. in 1979a who used Teflon tubing to the same effect. This design was used as part of a post-column extraction system. They concluded that the phase separator was a major contributor to band spreading.

The availability of Teflon filter membranes and porous plugs has resulted in their exploitation in a number of designs described between 1979-1989. They all depend upon the organic phase wetting and passing through the Teflon membrane or plug which is enclosed in a small volume chamber that rejects the aqueous phase. Notable among these was the design of Kwase et al., (1979) who were the first to use membrane separators for phase separation. In fact they used two phase separators in series, the first one was that designed by Karlberg and Thelander as discussed earlier and the second one was a porous PTFE membrane phase separator provided with a stirring device. This arrangement was used as they experienced leakage of aqueous segments into the detector, and the inability of the separator to handle aqueous phases which contained more than 10% methanol. This combined separator was able to handle aqueous phase which contained up to 40% methanol with chloroform as the organic phase. The stirring device in the separator was intended to decrease dispersion as it reduced the inner volume of the separator. The peak height was sharper when the bar was rotated than when it was static.

Nord and Karlberg (1980) used a PTFE membrane with polyethylene backing which was sandwiched between two pieces of perspex as a phase separator. The orientation of the phase separator depended upon the density of liquid phase which was being monitored. They

tested their separator with various water immiscible alcohols, alkanes, chlorinated hydrocarbons and aromatic solvents against water or water+methanol (1+1) and achieved a separation efficiency (the fraction of organic phase reaching the detector) of 0.8-0.9. They also found that as separation efficiency is increased peak height increased. The dispersion decreased about 30% when separation efficiency was increased from 0.4 to 0.9. In comparison with the separator designed by Karlberg and Thelander this separator gave lower dispersion. This separator was also designed for use with flow injection analysis.

Apart from the two membrane phase separators mentioned above various other similar designs appeared: Imasaka et al., 1981, Ogata et al, 1982, Fossey and Cantwell 1983, Burguera and Burguera 1983, Bigley et al. 1984, and Blackstorm et al 1985.

The phase separator designed by Ogata et al., was claimed to cause less dispersion than the one designed by Nord and Karlberg (1980) because of its small surface area. Bigley et al (1984) designed a phase separator which was used for extraction and detection of some phthalate esters using a post-column extraction system with LC-MS. The phase separator was tested with various percentages of acetonitrile and methanol (0-60%) in buffer or water as aqueous phase and chloroform as organic phase. For equal phase volumes (1:1) a separation efficiency of 0.4 was achieved for both sets of aqueous phases, however when the phase ratio was half organic i.e. 1:0.5 (aq-org) a separation efficiency of 0.2 (ACN-water) and 0.09 (MeOH-water) was achieved.

Burguera and Burguera (1983) used impregnated papers instead of Teflon. In the phase separator reported by Backstorm et al., (1985) the membrane was supported on a Teflon coated support. This support allowed the membrane to withstand high pressures. A separation efficiency of about 1.0 was achieved for all phase-volume ratios tested in the range of 1 to 30, using an aqueous phase consisting of 0.1 NaCl and chloroform or Freon - 113 as the organic phase.

Quinn (1989) tested various designs of membrane phase separators which were based on the design of Backstorm et al.,. These designs varied in their internal volume (3.2µl-117µl). The best results i.e maximum SE (95%) and lowest dispersion was achieved by using a

phase separator whose internal volume was 10 µl. It has been reported (de Ruiter et al.,1987) that biological fluids damage these membranes, thus reducing their useful working period.

In 1977 P.Copsey (1977) described a "microcell on-line extractor for biological fluids" that exploited the dissimilar wetting properties of water and organic solvents towards hydrophilic (glass) and hydrophobic (PTFE) surfaces. This approach was adopted by Kinkel and Tomlinson who described a design in 1980 (shown in Fig 3.5) that consisted of a single separating channel, and demonstrated its suitability for the rapid determination of drug partition coefficients and ion-pair extraction constants. No Teflon membrane or porous plug was needed in this design, so that its performance did not deteriorate with use. This phase separator was made available to Dr T.M.Jefferies for testing as an HPLC post-column phase separator, and was found to be capable of delivering clean organic phase to the detector at aqueous/organic flow rates of 1 ml min^{-1} each (Seymour, 1982), but it was subject to leakages and also to the irregular break-through of water droplets into the organic phase. In order to overcome these difficulties a separator was designed by Jefferies that possessed three long channels instead of one short channel as shown in Fig 3.6 (Badiru,1989). The aqueous and organic phases were mainly separated in the central channel and then each phase could be separately 'cleaned' in separate channels, simultaneously. This was a novel approach and it increased the separation efficiency from 0.6 to 1.0. Unfortunately, like the Kinkel and Tomlinson design it leaked under pressure. In 1987 deRuiter et al. described the evaluation of several variations on a design that consisted of two stainless steel blocks with a thick PTFE gasket held together by four bolts. Each design possessed a single, short, separating channel with small (30-45µl) internal volumes and providing separating efficiencies of 0.30-0.35 at 1 ml min^{-1}. The separator was tested with several aqueous (ACN/Water and MeOH/water mixtures) and organic (dichloroethane and n-heptane) solvents. The contribution of the phase separator to dispersion was not calculated separately. A miniature design was also made with an internal volume of 8 µl that was suitable for narrow bore HPLC at 0.2 ml min^{-1}. These designs are commercially available from Free University, Amsterdam.

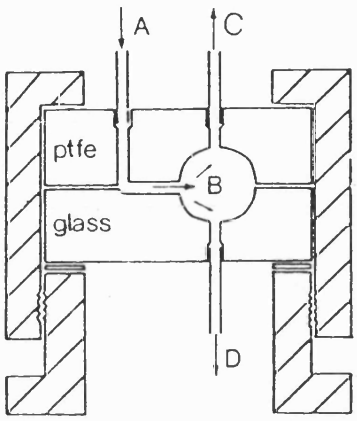


Fig 3.5 Phase Separator (Kinkel & Tomlinson, 1980) A= inlet, B=splitting chamber, C=oil phase, D= outlet

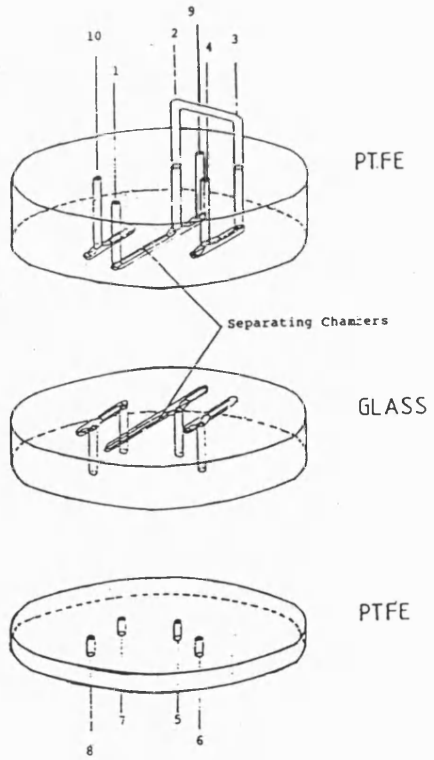


Fig 3.6 Phase separator(Badiru & Jefferies, 1989)

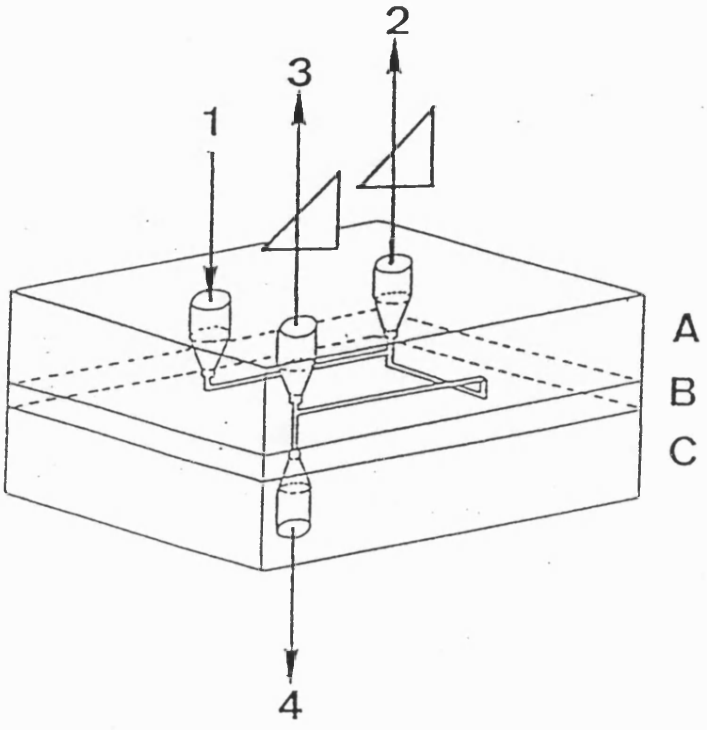


Fig 3.7 Phase separator design
1= input of HPLC eluent segmented by immiscible organic phase, 2= output of extracted HPLC eluent plus droplets of organic phase, controlled by SGE microneedle valve, 3= output of droplets of HPLC eluent in organic phase, 4= output of organic phase without water droplets, to the detector.

The detailed diagram of the phase separator designed by Jefferies and made by SSI (Scientific Systems Inc., State College PA, USA) is shown in Fig 3.7. and consists of two stainless steel blocks (A and C) and a central PTFE block (B) held together by four bolts. Grooves were cut into both the stainless steel and PTFE blocks to produce two channels having steel and PTFE surfaces, with a total internal volume of about 100µl. Each channel has an exit controlled by a MCV-50 micro-needle valve (Scientific Glass Engineering (UK) Ltd, Milton Keynes).

3.8 Results and discussion

As discussed above in Section 3.6, the ideal requirements for a phase separator are:

1. A separation efficiency close to unity for the maximum number of aqueous / immiscible organic mixtures.
2. Minimum dispersion
3. Robust and easy to use

The phase separator designed by Jefferies was evaluated on these three points

3.8.1 Separation efficiency

The performance of the phase separator was evaluated in the configuration shown in Fig 3.1 but without the HPLC column or detector. The separation efficiency was measured with various aqueous phases at different flow rates as shown in TABLE 3.1 with chloroform as the organic phase. All the aqueous phases contained the green dye, Screened Methyl Orange.

Needle valve 2 was adjusted to permit the release of organic phase plus a small volume of the aqueous phase, then needle valve 3 was adjusted to permit the release of remaining aqueous phase plus the minimum volume of the organic phase. Measurements of clean (completely free from any green aqueous droplets) organic phase were then made for at least an hour into a covered measuring cylinder. Separation efficiency (SE) was then calculated which is defined as:

$$SE = \frac{\text{Volume of organic phase through outlet 4}}{\text{Total volume of organic phase entering the detector}}$$

The range of aqueous to organic flow rates studied were as shown in TABLE 3.1

TABLE 3.1

Separation efficiencies using aqueous MeOH or aqueous ACN with chloroform as the organic solvent

Flow rates	Separation Efficiency								
Aq/Org ml min^{-1}	Water	MeOH %				ACN %			
		10	20	40	60	10	20	40	60
1.0/1.0	0.98	0.98	0.87	0.82	0.80	0.96	1.10	0.96	0.94
2.0/1.0	0.99	0.78	0.96	0.70	0.85	1.03	1.06	1.10	1.0
1.2/0.6	0.90	0.99	0.93	0.75	*	*	*	*	*
0.5/1.0	0.99	1.0	0.90	0.83	0.95	0.96	1.06	1.06	1.10
0.7/1.4	1.00	0.95	0.93	0.70	0.85	0.97	0.95	1.04	1.04

It was found that SE values of 0.8 were readily obtained and in the case of acetonitrile, often reached 1.10. This is possible because of extraction of acetonitrile into the chloroform. This has also been found by other workers e.g (Bigley et al., 1984). Above 70% (v/v), both methanol and acetonitrile are completely miscible with chloroform. and so 60% (v/v) is the practical limit. The orientation of the phase separator had no effect upon its performance, in that it worked equally well upside down, indicating that the separation effect was due to the wettability of the steel and PTFE surfaces towards the aqueous and organic phases, respectively. Density differences between the two phases were not necessary.

3.8.2 Post-column band spreading

(A). A measure of the individual contributions to band broadening made by the segmentation Tee connector (8, Fig 3.1), extraction coil (10, Fig 3.1) and the phase separator when employed in the arrangement as shown in Fig. 3.1 but without the components 3-7 was attempted with two systems.

Using acetonitrile-water (20:80,v/v) as the aqueous phase with 1,2-dichloroethane as the organic phase, at aqueous/organic flow rates of 1.0:1.0 ml min^{-1}, the separation efficiency was adjusted to 0.8. Injections of a biphenyl solution (20µl, n=10) were then made and the peak widths were measured at 10% peak height. This was repeated firstly after removing the coil, and then the phase separator. The results are shown in TABLE 3.2

TABLE 3.2 Contributions of various components of post-column system to band spreading. ACN-water with 1,2 - dichloroethane as organic phase, SE=0.8

Injection Valve	Tee piece	Coil	Phase Separator	Detector	Band width mm	sec
+	+	+	+	+	2.24±0.16	13.44
+	+	-	+	+	1.88± 0.10	11.28
+	+	-	-	+	1.20± 0.00	7.20
+	-	-	-	+	0.90± 0.10	5.40

(+) present (-) absent

With all the components in place, the mean initial band width was 13.4 sec, of which the coil contributed 2.16 sec, and the phase separator 4.08 sec. This study was repeated at an SE of 0.9 with heptane as the organic phase. The results are shown in TABLE 3.3. Under these conditions for an initial band width of 11.4 sec the coil contribution to band spreading was 1.14 sec, phase separator 3.46 sec. However when the same experiment was repeated at SE of 0.4 the band width increased from 11.4 to 20.3 sec, the contribution of the coil and the phase separator was 3.98 sec and 9.7 sec, respectively as can be seen from TABLE 3.4

TABLE 3.3 Contributions of various components of post-column to band spreading with heptane as the organic phase. SE=0.8

Tee piece	Coil	Phase separator	Detector	Band width mm	sec
	+	+	+	1.9 ± 0.15	11.4
+	-	+	+	1.71 ± 0.105	10.25
+	-	-	+	1.13 ± 0.15	6.8

TABLE 3.4 Contributions of various components of post-column to band spreading with heptane as the organic phase. SE=0.4

Tee piece	Coil	Phase separator	Detector	Band width mm	sec
+	+	+	+	10.15 ± 0.0	20.3
+	-	+	+	8.16 ± 0.362	16.32
+	-	-	+	3.3 ± 0.105	6.6

In both the solvent systems tested the contribution of the phase separator to band spreading was 3.46 to 4.08 seconds at a separation efficiency of 0.8. The phase separator performed similarly for hexane.

Reuter et al in their published paper have reported a separation of 0.4 - 0.5 and have recommended that their phase separator should not be used above this value. They did not calculate the contributions of various components of the phase separator.

B. Effect of Separation Efficiency on band width and peak height

In view of the observed effect of separation efficiency, its effect on peak height and peak width was examined in detail.

For this study four solvent systems were studied:

A. Acetonitrile- water (20:80 v/v) / heptane

B Acetonitrile-water (20:80 v/v) /dichloroethane

C. Methanol-water (70:30 v/v)/dichloroethane

D Isopropyl alcohol - water (20:80 v/v)/ heptane

The results for all the solvent systems showed that the relative peak heights for all the solvent systems increased from about 70 to about 90% when the separation efficiency was varied from 0.4 to 0.80 (Fig 3.8). Broadly similar results were obtained by DE Ruiter et al., (1987). Band widths in all the four systems decreased from 14-26 to 9-17 s when separation efficiency was raised from 0.4 to 0.80 in all the four systems (Fig 3.9). With biphenyl as the test solute, band width varied with the solvent system used. The choice of organic phase is influenced by the solubility of the analyte in the organic phase. For post-column ion-pair extraction, the nature of the analyte-ion-pair complex will determine peak heights in the various organic solvents. Under the experimental conditions used, dichloroethane and heptane proved useful solvents.

The effect of separation efficiency on peak shape can be seen in Fig 3.10. The decrease in band width at higher separation efficiency is considered to be due to the increased linear velocity of the organic phase through the detector flow cell

It was possible to run the phase separator at the maximum separation efficiency in all the four systems for as long as desired without the danger of leakage of aqueous segments into the detector

TABLE 3.5 give some phase systems in which phase separation is possible with the present phase separator.

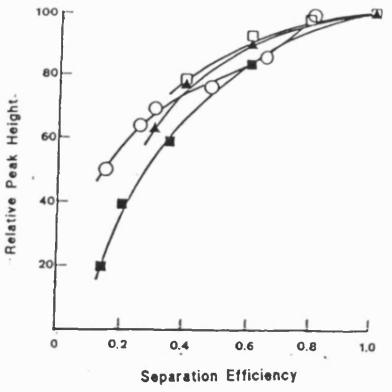


Fig 3.8 Effect of Separation efficiency (SE) on peak height ○ = A; □ = B; ▲ = C; ■ = D,the letters refer to solvent system as specified in the text

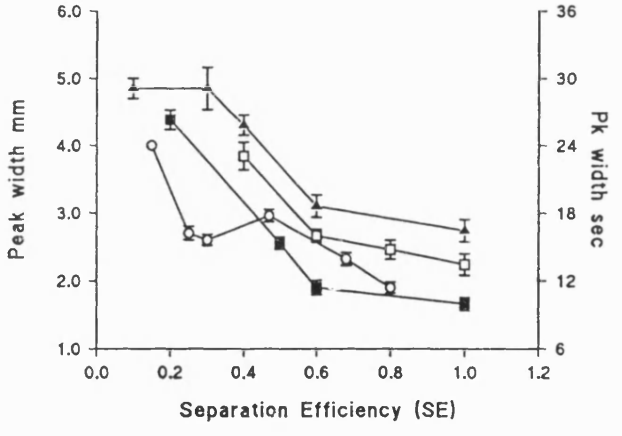


Fig 3.9 Effect of SE on peak width, codes as used for Fig 3.8.

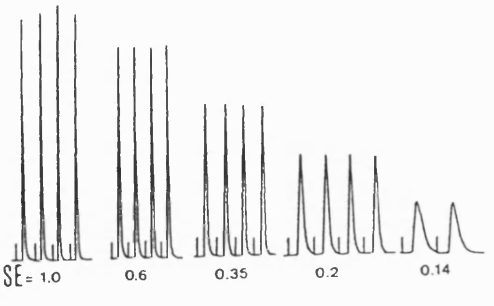


Fig 3.10 Effect of S.E on peak shape.

TABLE 3.5 Some of the solvent systems tested for evaluation of the phase separator.

Non-polar phase	Polar phase	Aq/Org	SE
Heptane	20% ACN-water	1.00	0.80
Dichloroethane	as above	1.00	0.99-1.00
Dichloroethane	MeOH-water	1.00	0.99-1.00
DCE + 10% pentanol	20% Isopropanol-water	1.00	0.99
Heptane	as above	1.00	0.99
Chloroform	water	1.00	0.99

The phase separator performed equally well for all the solvent systems tested without any major problems.

C.Contributions of injection volume to band spreading

De Ruiter et al. (1987) had shown that the injection volume contributed to band spreading (10 µl to 0.56 µl). To check whether injection volume contributes to band spreading in the present set-up System A i.e., ACN-water (20-80 v/v) / Heptane was used with an SSI injection valve (3XL HPLC injection valve -which has an internal loop system and has three internal loops 0.2 µl, 1.0 µl and 10 µl). Biphenyl was used as a test sample and dilutions were made in heptane so that each injection volume had the same weight of the compound i.e.,

0.2 µl (a) 0.38 mg/ml

1.0 µl (b) 1 in 5 dilution of (a)

10.0 µl (c) 1 in 10 dilution of (b)

The samples were injected (n=10), peak heights and peak widths were measured. The results are presented in TABLE 3.6. The results showed that under the experimental conditions used the injection volume did not significantly contribute to band spreading. The reason for this may be that about 24-26 segments are entering the coil and the calculated volume of each segment is 1-2 µl, therefore there is no difference between 0.2 µl and 1.0 µl.

TABLE 3.6

Injection volume	0.2 µl	1.0 µl	10 µl
Peak height (mm)	67.35 ±5.01	64.55 ±1.14	61.45 ±3.85
Peak width (mm)	1.78 ±0.14	1.72 ±0.08	1.64 ±0.85

The reason why there is no significant difference between a 10 µl injection volume and the rest is that with the whole system in operation and with the flow rate used (2 ml min^{-1}), the band spreading from the whole system overshadows the small difference the injection volume makes to band spreading.

3.8.3 Segment Stability

Solvent segmentation reduces not only band spreading but also acts as an extracting medium for the extraction of lipophilic ion-pairs e.g in ion-pair extraction system. To have reproducible results the number of segments entering and leaving the coil should be identical (Ruzicka and Hansen, 1981). Previous studies (Badiru 1989) had shown that when coiling is introduced to increase extraction, the centrifugal forces acting on the centre of the PTFE tube cause the alternate segments to coalesce into a larger segment so that the number of segments per centimetre leaving the coil is less than those entering the coil. This coalescing of segments not only causes unacceptable band spreading but also gives non-reproducible results. It is important therefore to find out what factors are responsible for segment stability. The study had shown that a coil diameter of 67 mm gave the best results.

For this experiment the same set-up as described in Section 3.8.1 was used. The injection valve was brought between the extraction coil and the phase separator. Water dyed green with 'Screened methyl orange' was used as an aqueous phase to visualise the segments with chloroform as an organic phase. Various flow rates (Aq/Org 1:1, 2:1 and 1:2) and various diameters of PTFE coils were tried i.e 30mm, 60mm and 85mm (0.8 and 1.0 mm i.d X 1.5m). After stopping the flows and turning the injection valve half way down, the segments were counted in the first 5cm and the last 5 cm of the PTFE extraction coil.

Preliminary studies indicated that under the experimental conditions used coiling had very little effect on segment stability. More important factors were the size of segments, which depend upon the internal diameter of the tubing bringing the organic stream into the segmentor, the internal diameter of the 'T' piece, and the pulsations in the pump. To stop the pulsations from the pump a 100 X 4.6 mm i.d column packed with 10 µ C_{18} was attached after the LO-PULSE dampener on the organic pump.

For example using flow rates varying from 0.80 ml min^{-1} to 4.2 ml min^{-1} (total flow rate aq/org) using a 1.0 mm i.d coil wrapped on a glass rod with a 1.08 mm i.d 'T' piece, the number of segments entering the coil and leaving the coil were approximately the same. Similar results were obtained when the same tube was clamped straight. The results on a coiled tube under the above conditions are shown below in TABLE 3.7.

TABLE 3.7 Tubing wrapped around a glass rod, T piece =1.08 mm.id.

Flow rate (Aq/org) ml min^{-1}	No of segments in (±s.d)	No of segments out (± s.d)
0.4	8	6.16 (0.983)
0.8	8.2 (0.836)	8.2 (0.836)
1.2	9.0 (0.707)	8.8 (1.64)
1.4	9.5 (0.577)	9.75 (0.5)
2.8/1.4	13 (0.00)	13.0(0.00)
2.0/1.0	13.00(0.00)	11.80 (1.64)
1.20/0.6	8.33 (0.816)	8.8 (1.09)

Changing the 'T' piece to the one with a small internal diameter (0.375 mm) and a 0.8 mm PTFE tubing showed that the segments are generally more stable (within experimental results) in straight tube (TABLE 3.8) than in the coiled tubes (TABLE 3.9).

TABLE 3.8 Straight tubing with 0.375 mm i.d T piece

Flow rate (ml min^{-1}) Aq/org	Number of segments in (± s.d)	Number of segments out (± s.d)
0.4	15.0 (1.58)	12.4 (1.81)
0.8	20 (2.82)	18.8 (5.22)
1.20	18.0 (1.224)	17.0 (1.87)
1.40	15.6 (0.408)	8.0 (3.03)
2.8/1.40	11.80 (0.83)	7.80 (1.30)
2.0/1.0	15.28 (2.42)	8.57 (1.90)
1.20/0.6	17.66 (1.03)	15.2 (2.68)
0.8/0.4	23.0 (2.68)	12.0(4.77)
0.5/1.0	8.80 (0.83)	7.8 (1.92)
0.7/1.4	16.6 (0.89)	13.5 (1.0)

TABLE 3.9. Tubing coiled around a glass rod, rest of conditions same as Table 3.8

Flow rate (ml min^{-1}) Aq/org	Number of segments in (± s.d)	Number of segments out (± s.d)	
0.4	13.8 (0.83)	9.22(2.68)	67
0.8	14.6 (2.50)	14.8 (2.94)	101
1.20	23.2 (0.447)	6.2 (0.44)	27
1.40	23.0(2.12)	7.2 (1.92)	31
2.8/1.40	15.2 (3.11)	12.4 (1.15)	82
2.0/1.0	17.5 (3.83)	10.33 (1.63)	59
1.20/0.6	14.4 (1.34)	13.4 (1.51)	93
0.8/0.4	16.83 (3.18)	16.66 (3.20)	99
0.5/1.0	12.66 (2.64)	12.00 (3.08)	95
0.7/1.4	14.00 (1.41)	12.50 (2.58)	89
0.8/1.0	14.66 (4.13)	12.00 (0.89)	82

However at low flow rates i.e at low linear velocities, segments are stable in both sets of tubes, which is to be expected because only at high linear velocities will the centrifugal forces increase sharply and cause the faster moving central fluids to be pressed against the wall thus decreasing the thickness of the boundary layer between the segments causing them to coalesce. (Tijsen,1980).

The reason why the segments are unstable in straight tubes at a total flow rate of 2.4 $mlmin^{-1}$ (1.2 ml min^{-1} Aq/Org) and 2.8 ml min^{-1} (1.4 ml min^{-1}) when they are stable at a total flow rate of 4.2 ml min^{-1} (2.8/1.4) cannot be explained by an increase in linear velocity, perhaps some factor other than linear velocity is responsible for segment stability e.g gravity (Cantwell and Sweileh,1985). In all these studies the effect of gravity on segmentation was not taken into account.

Initial experiments in this section were carried out using a 'T' piece with an internal diameter of 0.345 mm. and 0.8 mm i.d coil, as it gave smaller segments which would increase extraction. However problems were experienced using the 'T' piece when it was used in combination with post-column ion-pair extraction of drugs of abuse (Section 3.8.6) because of pulsating base line noise, which disappeared when this 'T' piece was replaced by a 'T' piece with a bigger internal diameter i.e 1.08 mm.

The PTFE extraction coil was later replaced with stainless steel tubing. Segment stability would have much greater influence on band spreading when PTFE coils are used than when

stainless steel coils are used. This is because of the 'wetting phenomenon' as explained earlier i.e in PTFE coils the aqueous segments move in the form of plugs and the organic solvent drags along the walls. In stainless steel coils the organic solvents travel as plugs while the aqueous phase drags along the walls

3.8.4 Influence of tube length and coiling on extraction

To measure the effect of tube length and coiling on extraction, a solution of caffeine (0.192 mg min^{-1}) in citrate phosphate buffer pH 4.0 was used as an aqueous phase at a flow rate of 1.0 ml min^{-1}, and chloroform which was previously equilibrated with citrate phosphate buffer (without caffeine) was used as an organic phase at the same flow rate. After the flow rates were stabilised for 15 minutes measurements were made off-line on both aqueous and organic layer. To determine the length of extraction coil required for efficient extraction, a PTFE tube of 0.8mm i.d was cut into lengths of 120 mm. 310 mm, 610 mm and 1245 mm and used in random order. To examine the effects of coiling the tubes were coiled around a 25 mm beaker except for the 120 mm length where a 8 mm test tube was used for making the coil. TABLE 3.10 shows the results for the tube length .

TABLE 3.10 Effect of tube length on extraction of caffeine.

Tube length	Volume of coil (ml)	Concentration in organic layer (n=3)	Concentration in aqueous layer
120 mm	1.50	0.160 (0.141)*	0.051
310	3.89	0.165 (0.186)	0.006
610	7.66	0.191 (0.181)	0.012
1245	15.63	0.199 (0.192)	0.00

* calculated value (0.192 mg ml^{-1}) - Concentration in aqueous layer

The results showed that as the length of extraction coil is increased, the extraction increased from about 73.4% for the first 120 mm of the tube, with the remaining 26.5% extracted in the remaining 1125 mm of the tubing. TABLE 3.11 shows the results when the same tubes were coiled as described earlier. As can be seen from TABLE 3.11 coiling marginally improves extraction at shorter tube lengths i.e. 120 mm, however the effect of coiling is not noticeable when longer coils are used. For practical purposes coiling was preferred for ease of use (except the 120 mm coil), all were made using a 50 mm. beaker.

TABLE 3.11 Effect of coiling on extraction of caffeine

Tube length (mm)	Aqueous layer (n=3) mg/ml	Organic layer (n=3) mg/ml
120	0.188 (0.144)	0.048
310	0.205 (0.179)	0.013
610	0.203 (0.182)	0.010
1245	0.209 (0.190)	0.002

To check further the optimum length of coil for the extraction of other compounds further experiments were conducted using the SE-FIA set-up shown in Fig 3.2 with phenobarbitone (log P 1.61 Clark) and sulphamerazine (log P -0.1) as test compounds using water and chloroform as aqueous and organic phase respectively.

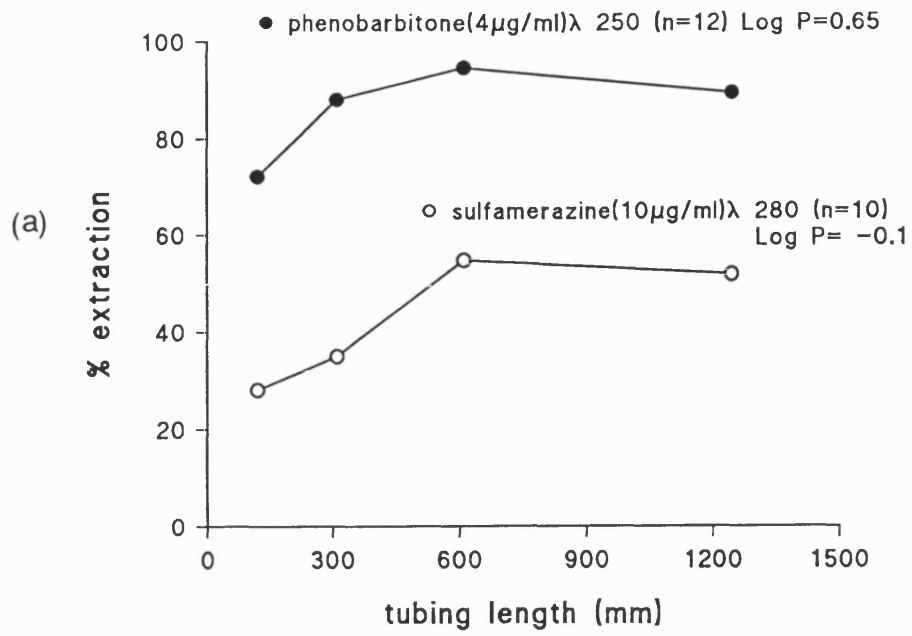
The solutions of both the substances were made in water and also in chloroform or chloroform-methanol. The aqueous sample was injected into the aqueous stream and the organic sample was injected into the chloroform layer (non-extracted solution) and the percentage extraction was calculated. by using the following formula:

$$\% \text{ extraction} = \frac{\text{peak area of aqueous solution}}{\text{peak area of non extracted solution}}$$

Fig 3.11(a) shows the results both for phenobarbitone and sulphamerazine. For both these compounds the maximum extraction i.e 94.5% for phenobarbitone and 54.64% for sulphamerazine was achieved using a 610 mm coil, further increase in coil length did not improve the extraction. The extraction of any compound and the sensitivity achieved depends upon its log P value. The peak width at half height for all the tube lengths varied between 1.8 - 2.0 mm, which showed that the contribution of the coil towards band spreading was not very significant.

As the purpose of these optimisation studies was to use post-column ion-pair extraction it seemed appropriate to test the system in that configuration for some amines. For this purpose Naphthalene 2-sulphonic acid (N2S) was used as an ion-pairing agent and phenylethylamine (PEA) and dexamphetamine were used as test compounds.

Experiments were performed using 0.01 M N2S in phosphate buffer pH 3.5 as an aqueous phase and chloroform + 10% pentanol as an organic phase at a flow rate



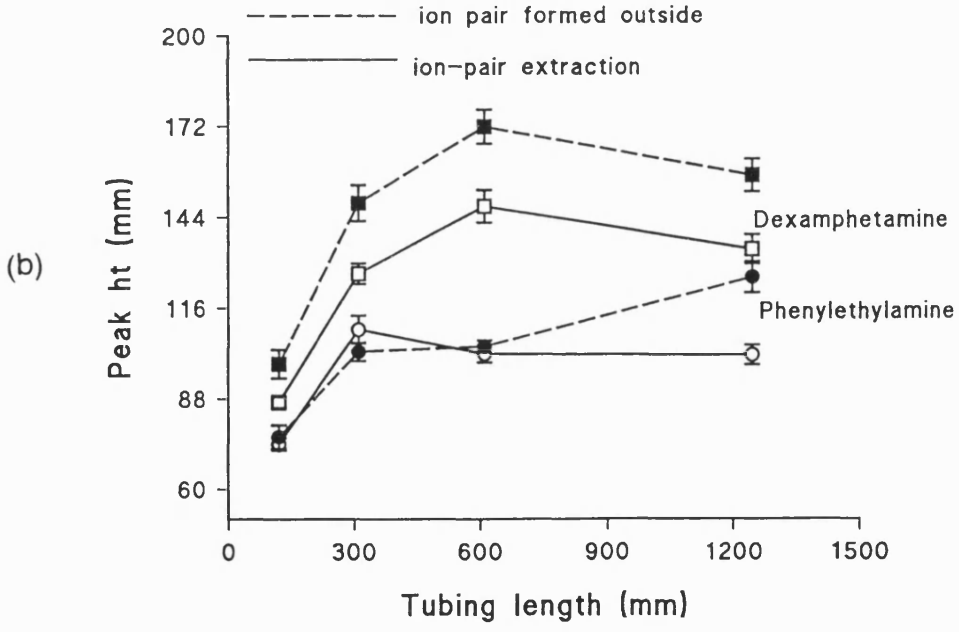


Fig 3.11 Effect of tube length on extraction

of 1.0 ml min^{-1} (the organic phase was equilibrated with the aqueous phase before being used). The SE-FIA system shown in Fig 3.2 was used for these studies. The solutions of PEA and dexamphetamine (DEX) were made in buffer (19.6 µg/ml for PEA and 0.328 mg/ml for DEX) and in the aqueous phase (preformed ion-pair) peak heights were recorded for both sets of solutions. Fig 3.11(b) shows the results of this study. The extraction for both the amines as N2S ion-pairs is maximum at 610 mm and then decreases slightly. The extraction of dexamphetamine ion-pair is better than that of PEA ion-pair which is to be expected as it is more hydrophobic than PEA. The minimum detectable limit under these conditions was 3.92 µg/ml for PEA and 1.32 µg/ml for dexamphetamine.

Naphthalene 2 sulphonic acid is a U.V ion-pairing agent and has a λ max of 272 nm which is very close to that of chlorinated organic solvents e.g chloroform. DAS is a fluorescent ion-pairing agent and λ max for DAS ion-pairs is 374nm and would therefore give less background signal. N2S was therefore replaced by DAS as an ion-pairing agent. Another advantage of DAS is that it is more hydrophobic than N2S and has a higher extraction constant than N2S i.e 3.45 for N2S (Schill 1974) and 5.75 for DAS (Westerlund and Borg 1973). Further work in this section and subsequent sections was carried out using DAS as an ion-pairing agent.

To investigate the use of DAS as a post-column ion-pairing agent the set-up shown in Fig 3.1 was used with a minor change that the injection valve was used between the analytical column and the 'T' piece. As the aim of the work was to develop a sensitive method for the detection of some drugs of abuse, pethidine, dipipanone and methadone were used as test solutes. An analytical method for methadone, cocaine and phencylidine from plasma has been reported in the literature (Derendorf and Garrett, 1983). The method used 0.025 M acetate buffer pH 3.6 containing 30 mg DAS L^{-1} as the post-column ion-pairing agent. The chromatography was performed on a cyano column, chloroform was used as an extracting solvent of lipophilic ion pairs. The sensitivity of the method using 1 ml of plasma (250µl) and injecting 100 µl of sample was 1-6 ng/ml.

Using DAS as a post-column ion-pairing agent with a PTFE extraction coil (1245 X 0.8 mm i.d.) the peaks were broad and tailing but the peak shape improved when the same dimension stainless steel coil was used. The reason for broad and tailing peaks was the 'wetting phenomenon' as discussed earlier.

Fig 3.12 shows some chromatograms obtained for dipipanone, methadone and pethidine using SE-FIA (three pump system).

The aqueous phase was 0.025M phosphate buffer-acetonitrile- isopropanol (76:12:12 v/v) at a flow rate of 0.5 ml min^{-1} (Badiru 1989). An aqueous solution of DAS 6 X 10^{-5}M as a post-column ion-pairing agent at a flow rate of 0.4 ml min^{-1} and dichloroethane 1.5 ml min^{-1} was used as an extracting solvent.

3.8.5. Influence of counter-ion concentration

As mentioned earlier the efficiency of a post-column ion-pair extraction system can be optimised by varying the concentration of ion-pair and varying the organic solvent used for the extraction of lipophilic ion-pairs (section 3.3). Increasing the concentration of the counter-ion generally increases the extraction of the ion-pair. The concentration of the counter-ion however depends upon its solubility in the aqueous solution and its solubility in the 'free form' in the extracting solvent. The latter increases the background noise thus compromising the sensitivity of the method. Furthermore, increasing the percentage of organic modifier in the mobile phase also increases the solubility of the counter-ion in the extracting solvent. The concentration of counter-ion therefore needs to be tuned for the specific extraction system e.g it will vary with the chromatographic system employed and whether an isocratic or gradient system was being used.

In trying to optimise the concentration of ion-pair it was also found that the optimum concentration of the counter-ion varied with the type of fluorescence detector being used e.g using a PE 204 S the DAS concentration was 1 X 10^{-4} M whereas using a JASCO HPLC fluorescence detector the concentration of DAS had to be lowered to 1X10^{-5} M or less.

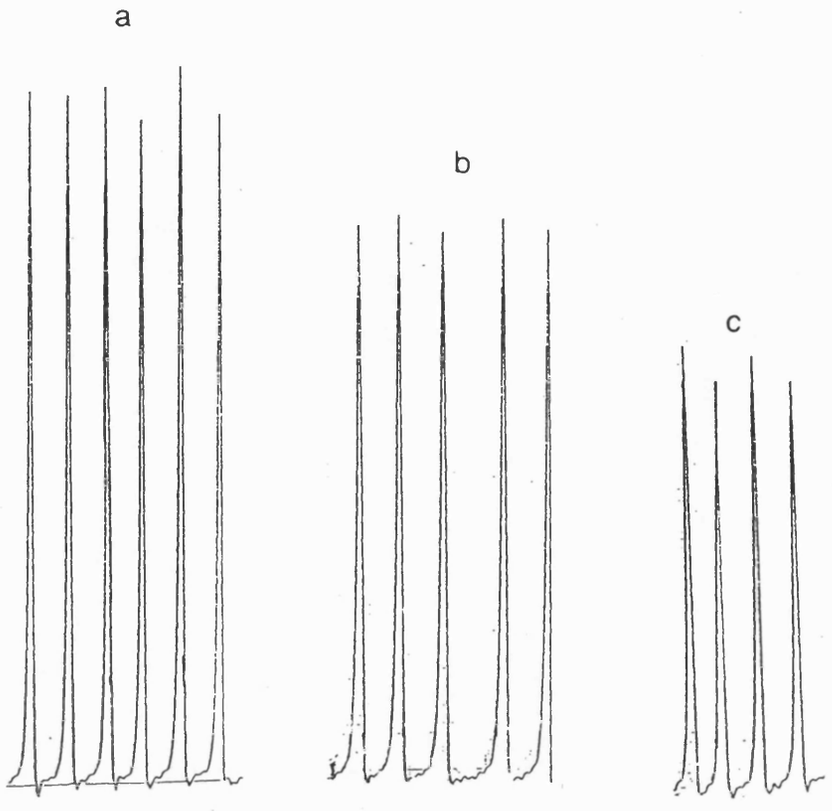


Fig 3.12 Testing of post-column system with some drugs of abuse (a) pethidine 1.7 µg/ml, (b) Dipipanone 2.0 µg/ml, (c) methadone 1.2 µg/ml.

To investigate the effect of counter-ion concentration a mobile phase consisting of acetonitrile-isopropanol-0.025 phosphate buffer pH 4.0 (76:12:12 v/v) was used at a flow rate of 0.5ml min^{-1} with a methyl-cyano column 100 X 4.6mm i.d (Phase Sep). The concentration of DAS was varied from 1 X 10^{-4} M to 6 X 10^{-6} M at a flow rate of 0.4 ml min^{-1}. Dichloroethane was used as an extracting solvent at flow rate of 1.5 ml min^{-1}. Studies were carried out using a Jasco HPLC detector.

DAS at a concentration of 1 X 10^{-4} M gave a high background and was therefore not appropriate. For practical reasons 3 X 10^{-5} M was considered appropriate as it gave the highest peak heights i.e 155±2.82 for pethidine compared with 111.5 ±1.0 (n=3) at 6 X10^{-6} M. The nature of organic solvent greatly influences the extraction of ion-pairs. Hydrophobic compounds containing H-accepting groups are generally well extracted by H-donating solvents like chlorinated solvents e.g dichloromethane, chloroform etc. Likewise hydrophilic compounds are well extracted by H-accepting solvents. The extracting ability of chlorinated organic solvents is insufficient for extraction of low molecular weight compounds that carry hydrophilic groups. This can however be improved by the addition of lipophilic alcohols e.g pentanol to chlorinated organic solvents (Schill, 1974) As an extracting solvent dichloromethane, dichloroethane and dichloroethane containing 5% pentanol were examined. The presence of pentanol gave a high back ground noise making its use difficult. Dichloromethane gave higher peak heights e.g 133 mm for pethidine as compared to 117mm with dichloroethane, but it also gave a higher background noise and the formation of air bubbles in the tubes. Dichloroethane was therefore preferred.

3.8.6 Application

As an application of post-column ion-pair extraction using DAS as a counter-ion a number of analytes were selected whose chromatography had previously been investigated at 205 nm (Badiru ,1989 and Section 4 of this thesis). These compounds were tertiary amines and have poor U.V detectability (apart from cocaine) and are difficult to derivatize. However they can be readily protonated and can therefore form ion-pairs with suitable counter-ions like DAS.

The compounds used in this study were pethidine, cocaine, methadone, normethadone, piritramide, and dipipanone. Normethadone was used as an internal standard in this application, however any of the substances could be similarly used. The analytes were separated on an SGE cyano column (100 X 2.1mm i.d.) using a step gradient at a flow rate of 0.2 ml min^{-1}. The mobile phase used was:

A; acetonitrile-isopropanolol-0.025M phosphate buffer adjusted to pH 4.0 (3-3-94 v/v/v)

B: same as A but with a higher percentage of acetonitrile and isopropanol i.e (25:25:50 v/v/v).

The gradient conditions were, 80:20v/v (A:B) for 5 minutes. At 5.1min 55:45 v/v (A;B) till 20 minutes and then back to 80:20 v/v (A;B). This was achieved using an SSI gradient controller in configuration 1 i.e without any modifications (for details see Section 2.2.3 modifications to gradient controller). DAS 8.85 X 10^{-5}M was used as the post column ion-pairing agent at a flow rate of 0.4 ml min^{-1}. Dichloroethane was used as an extraction solvent at a flow rate of 0.6 ml min^{-1}.

Fig 3.13 shows some sample chromatograms of highest (a) and lowest concentrations (b) obtained using the above conditions. The calibration plots for all the 5 components were linear in the concentration range tested and are shown in Fig 3.14. A step gradient was needed to separate the early eluting peaks from the sample interfering peaks and at the same time elute the more hydrophobic analytes as sharp symmetrical peaks.

Fig 3.15 (a-b) and Fig 3.16 (a-b) show some of the chromatograms obtained for blank and spiked urine and plasma at 205nm, and under post-column conditions using Bond Elut Certify cartridges (manufacturers procedure for amphetamines). As can be seen from the chromatograms post-column system not only offers increased sensitivity over 205 nm but also gives a cleaner chromatogram.

Due to the technical problems encountered with the gradient controller, and the presence of 'interfering peaks', probably due to the Vac Elut 24 system attempts to optimise the extraction procedure for better clean up of plasma and urine samples

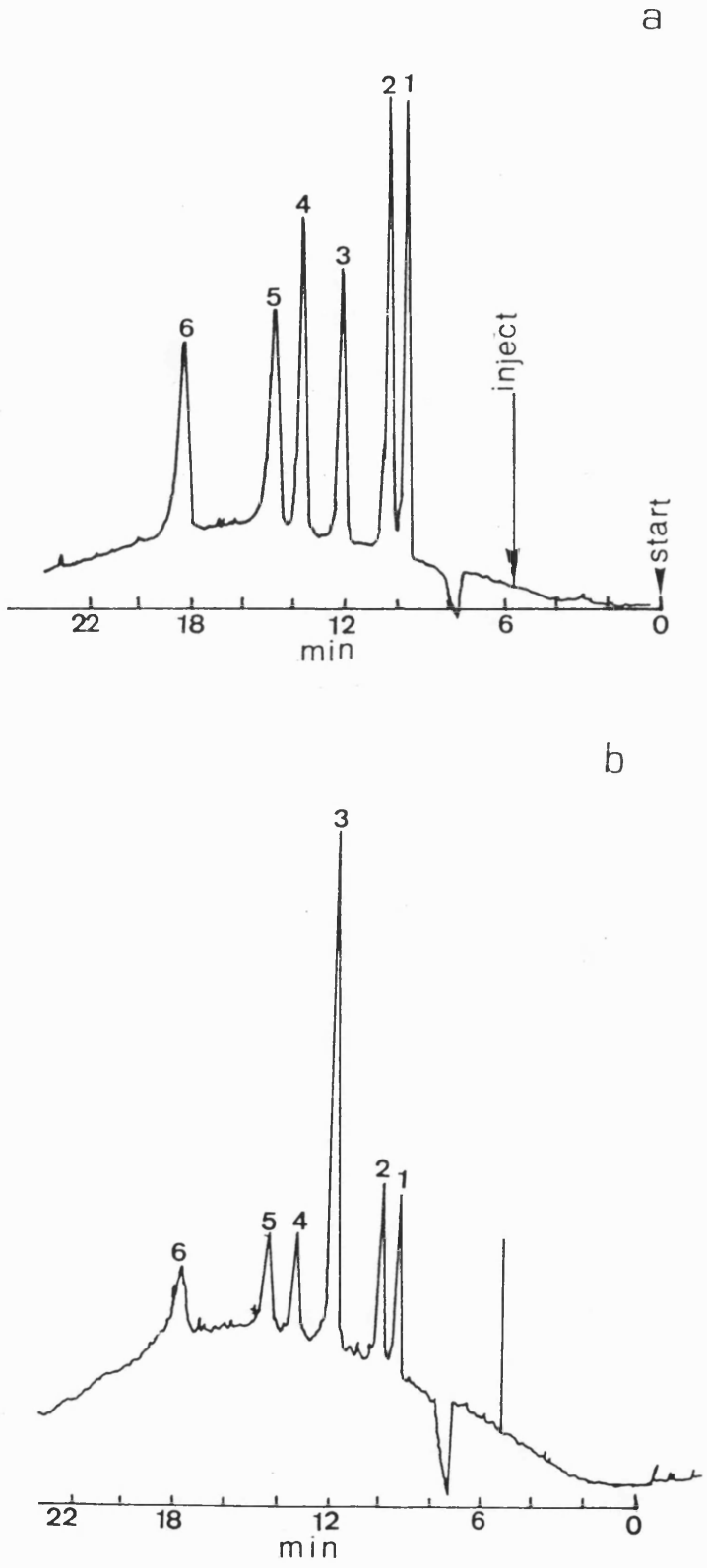


Fig 3.13 Sample chromatogram of highest and lowest concentration of selected drugs of abuse, conditions as specified in the text. (1) pethidine, (2) cocaine, (3) normethadone, (4) methadone, (5) piritramide, (6) dipipanone. (a) 4-9 ng on column, (b) 0.8 - 2.0 ng on column using a 10 µl loop.

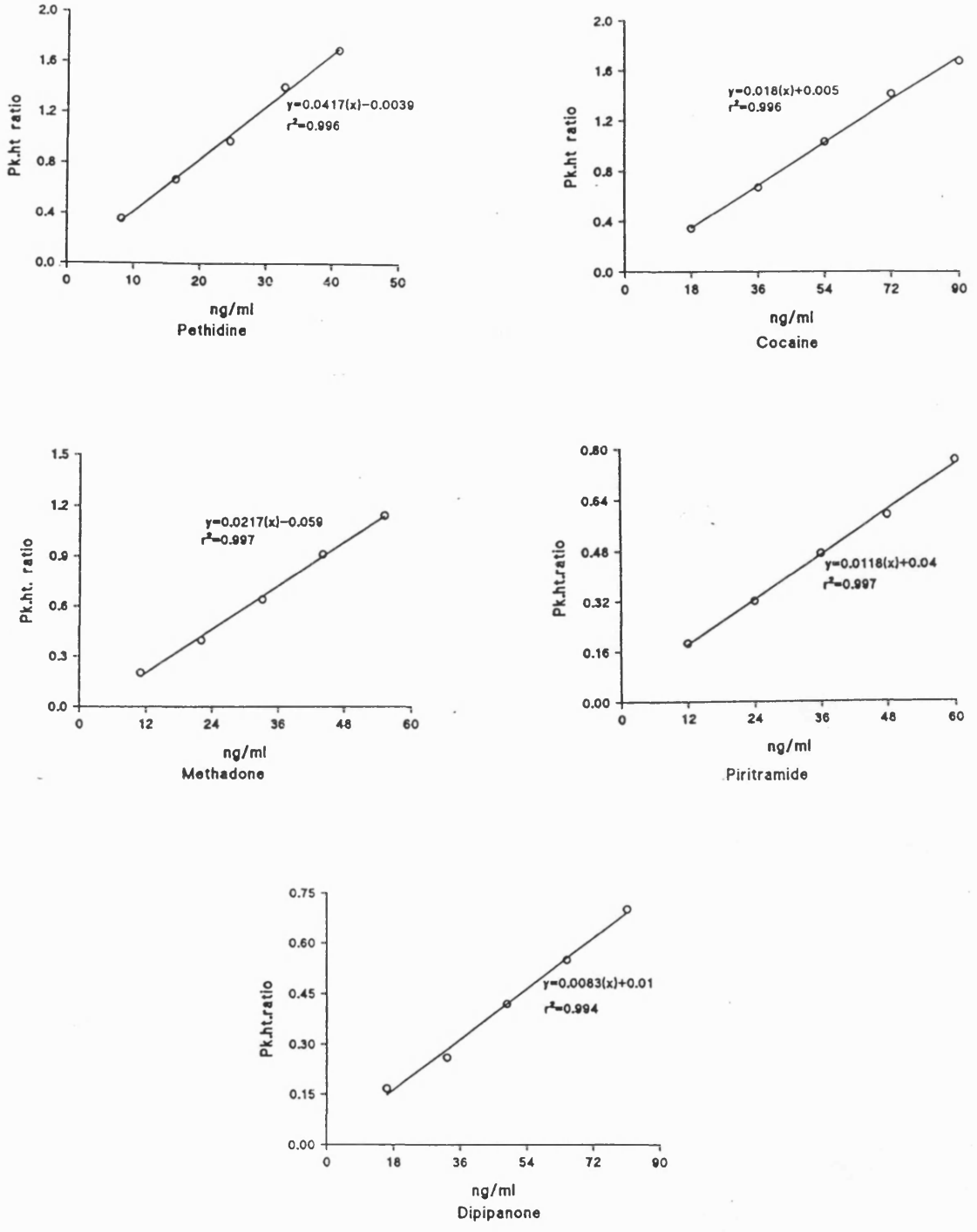


Fig 3.14 Calibration plots of standard solutions of selected drugs of abuse, as specified.

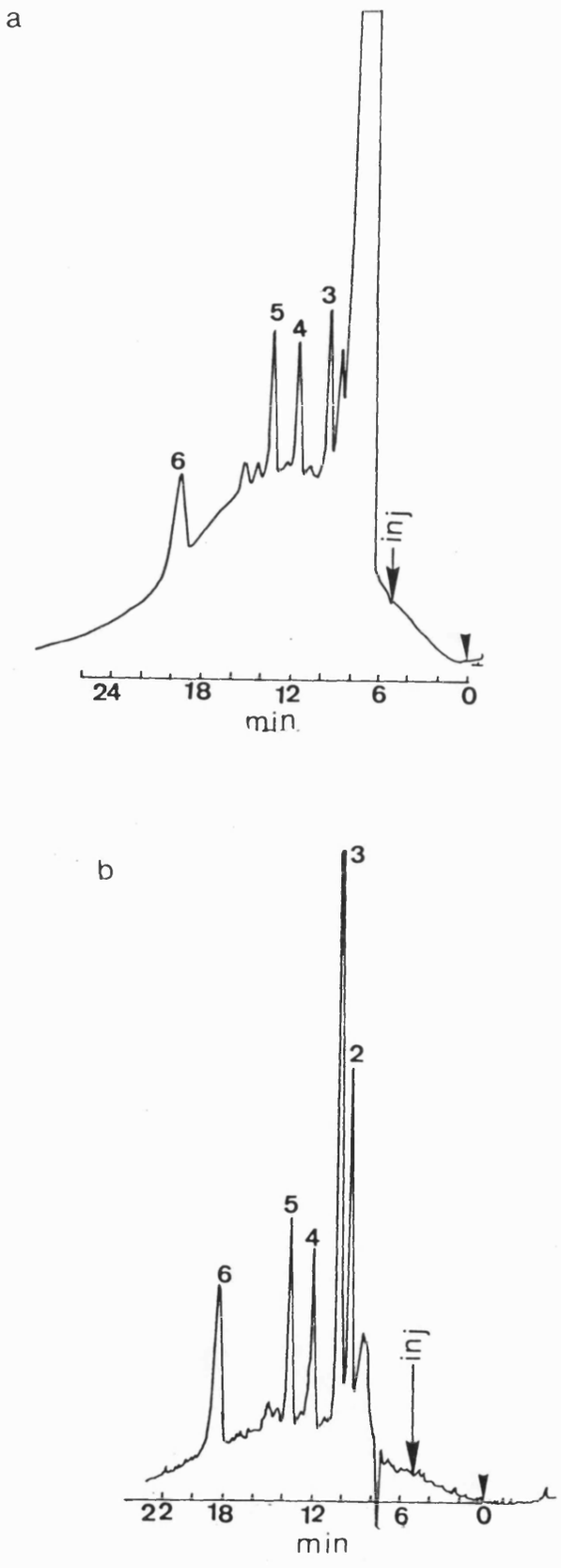


Fig 3.15 Chromatogram of spiked urine after SPE (a) 205 nm., (b) post-column, codes same as Fig 3.13.

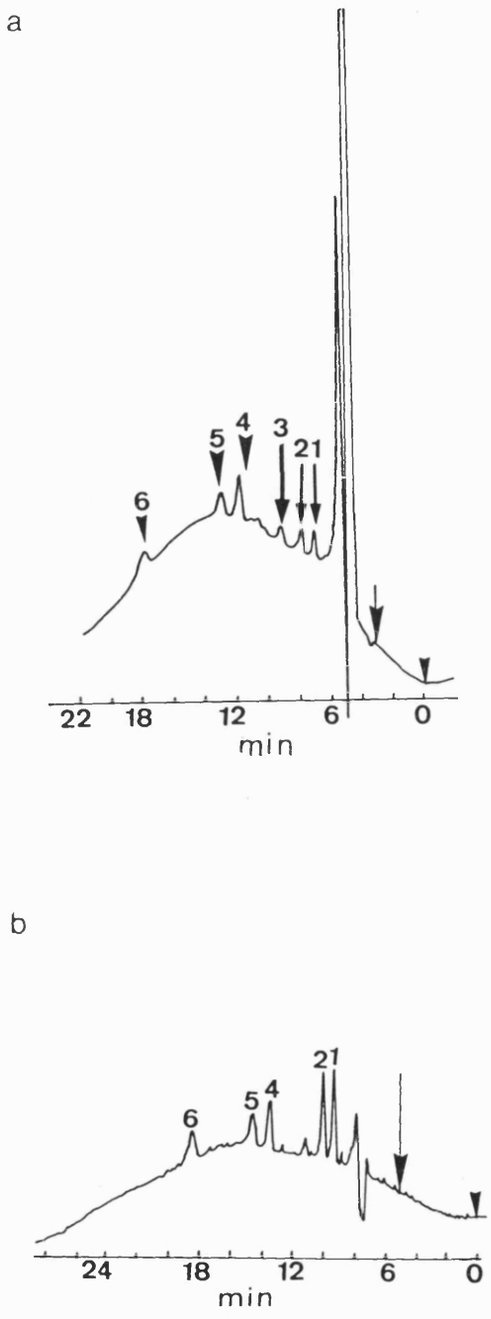


Fig 3.16 Chromatogram of spiked plasma after SPE (a) 205 nm., (b) post-colum, codes same as Fig 3.13.

was not performed.

3.9 Conclusions

In this section the performance of a phase separator designed by Jefferies and made by SSI (USA) has been evaluated with various solvent systems for (a) separation efficiency, (b) band spreading and (c) ease of use.

With all the solvent systems tested it was possible to achieve a separation efficiency of unity. The contribution of the phase separator to band spreading depends upon its separation efficiency. At a separation efficiency close to unity the contribution of the phase separator to band spreading is between 3.5 to 4.0 seconds. The phase separator required little attention during use as long as other components of the system i.e pumps were working normally. Under the experimental conditions the injection volume did not make a significant contribution to band spreading.

This work has also demonstrated that gradient conditions can be employed for post-column ion pair extraction systems using narrow bore columns. The post-column ion-pair extraction system offered a four fold increase of sensitivity over 205 nm (2ng on column) and is less affected by interfering peaks from the sample matrix.

Some of the problems which were encountered in this work were the need to modify a low pressure gradient system for gradient elution for use with narrow bore columns at flow rates of 0.2-0.4 ml/min without a significant delay time i.e 25 minutes (as was in this case) and the presence of interfering peaks from the solid phase extraction system.

4 OPTIMISATION OF CHROMATOGRAPHIC SYSTEM

4.1 Introduction

An important requirement for the effective use of extraction detectors is that the chromatographic mobile phase must be compatible with the post-column reaction conditions. For example Farinotti et al. 1983 found that the fluorescent intensity of derivatized fatty acids with 4-bromomethyl-6,7-dimethoxycoumarin was strongly dependent on the HPLC mobile phase. It was highest in water (0.64) and decreased as the percentage of methanol or acetonitrile was increased (0.43 in methanol, 0.20 in acetonitrile) thus limiting the use of derivatization to isocratic separations. Gfeller et. al., (1979) also found that with DAS as counter-ion the intensity of the detector signal reduced as the methanol content of the aqueous phase was increased. In both cases the presence of methanol increased the background signal which adversely affected the sensitivity of the method. Some of the requirements for chromatographic mobile phases for post column ion-pair extraction detectors are:

1. Analytes must give sharp symmetrical peaks. This is important because no matter how well a post-column system is designed, some additional band spreading will occur and this is especially important for late eluting peaks. The band spreading caused by the post-column reactor can be compensated by sharp eluting peaks from the column..
2. The use of high ionic strength buffers or mobile phase additives such as silanol suppressing agents increases the background signal and should therefore be avoided.
3. The chromatographic mobile phase should be predominantly aqueous, as increasing the organic modifier in the mobile phase increases the background signal thereby reducing the sensitivity of the method.

The analytes which were studied in this thesis were compounds of low molecular weight, water soluble, polar and ionisable. Reversed phase high performance liquid

chromatography (RP-HPLC) was therefore considered to be an appropriate choice for the chromatographic separation of these compounds.

The most commonly used materials for RP-HPLC are alkyl bonded silica phases e.g C_8 and C_{18}. The availability of small particle sizes (3-5μ) and efficient packing techniques have enabled manufacturers to market columns with very high efficiencies. The bonded phases show not only a wide variation in selectivity when the same phase e.g C_{18} is obtained from different manufacturers but they also show variation from batch to batch even when obtained from the same manufacturer. It is often very difficult to get the same separation on a column which is different in batch number from the one on which the method was originally optimised. These differences are due to the nature of silica and the bonding procedure used. TABLE 4.1 shows the wide variety of silica used by manufacturers to make alkyl bonded phases (Antle et al. 1985).

TABLE 4. 1

Pore size (nm)	6-30
Surface area	100-500 m^2/gm
pH of silica	4-10

Some of these variations in the nature of silica can be exploited to give beneficial results as discussed later in this section

The bonding procedure is necessary to change the polarity of the silica surfaces so that it can be used with polar mobile phases e.g water. However because of steric hindrance during the bonding stages only partial alkylation of the silanol groups of the silica surface takes place and residual silanol groups remain on most columns (Sander,1988). The presence of these residual silanols give the columns split personalities i.e., non polar molecules interact primarily with the C_{18} functions (hydrophobic interactions) and elute as sharp symmetrical peaks, whereas sample molecules e.g. amines that are attracted to SiOH groups (silanophilic interactions) interact both with the C_{18} as well as SiOH functions and elute as badly tailing peaks.

The tailing peaks are problematic as they can cancel out any gains which are made in selectivity, retention or band width by using highly efficient columns or by careful

optimisation of the mobile phase. It is important to point out here that most manufacturers test their columns with neutral solutes under ideal conditions so as to 'break world records in plate numbers' (Engelhardt and Jungheim, 1990). A manufacturers test chromatogram may not be the best guide to how a column will perform during actual analysis e.g. the separation of two peaks with base line resolution and perfect peak shape may be useless if the first peak tails badly (Dolan, 1989).

4.2 Literature review of Analytical Methods

A variety of methods have been suggested for the chromatography of basic compounds. Papp and Vigh (1983 a,b) proposed the use of high ionic strength buffers to minimise the ion-exchange interactions between protonated bases and ionized silanols. Knox and Hartwick, (1981) used ion-pair chromatography, this approach increases the retention of solutes but frequently does not improve peak shapes (Lingeman et al 1986). Chromatography of analytes as neutral molecules at a pH chosen to suppress ionization e.g reversed phase separation of weak acids such as Δ–tetrahydrocannibinol using a mobile phase consisting of methanol-0.02M H_2SO_4 (4:1) (Snyder and Kirkland, 1979). This approach reduces the life of the column as alkyl bonded silica phases can be used only between pH 2-8.

The use of polymeric columns has also been suggested (Yasin and Jefferies, 1988) as they lack silanols and are stable at pH range of 1-13, and therefore can be readily used for chromatography of basic compounds at alkaline pH (8-12.5). Although the polymeric columns give symmetrical peak shapes for most compounds, the chromatography is not very good because these columns have poor efficiency due to slow stationary phase mass transfer (Badiru,1989).

The most common method to prevent tailing of basic analytes is the addition of silanol suppressing agents to the eluent e.g triethylamine for bases and acetic acid for acids. They prevent tailing because they interact more strongly with surface silanols than the

sample molecules. According to Lingeman et al (1986) the use of such silanol suppressing agents shortens column life.

Synder et al., (1988) have suggested that due to wide variations in the pH of the original silica different brands of column should be investigated for different compounds as some columns give excessive tailing for basic compounds. For example Zorbax Rx was found to be most suitable for basic compounds but very bad for acids, alternatively Micropak was found to be very efficient for acids but was bad for bases. These differences occur because of differences in the percentage of various types of silanols (acidic and basic) on various brands of columns. Over the last few years various manufacturers have designed special base-deactivated columns for the chromatography of basic compounds e.g. Supelcosil DB (Supelco), Hypersil BDS (Shandon), Suplex pKb-100 (Supelco) and Supelco ABZ. The ABZ stands for acids, bases and zwitterions.

Apart from the above methods, non-modified silica using methanol-ammonium nitrate (90:10 v/v) at basic pH (9-10) has been successfully used for basic drugs of forensic interest i.e., drugs of abuse (Jane, 1975) with very good peak shapes and high column efficiencies i.e around 70,000 plates/metre (h=2-3) (Law, 1987). Hansen et al. (1987) investigated the chromatography of nitrogen containing bases on chemically bonded ODS silica, deactivated ODS and dynamically modified silica, where chromatographic separation was performed on a non-modified silica column with an eluent containing a long chain quaternary ammonium compound i.e cetyltrimethylammonium bromide at pH 7.0. The deactivated and ODS phases exhibited tailing problems. However the tailing was slightly less with the deactivated phase. In both cases it was reduced by addition of anti-tailing agents. On dynamically modified silica 'the tailing was negligible'.

Although the use of non-modified silica has found wide-spread use for the chromatography of drugs of abuse of forensic interest, unfortunately it is not suitable for post-column ion-pair extraction detection as the mobile phase contains a high percentage of organic modifier i.e around 90% and was therefore not pursued.

De Smet and Massart, 1987 investigated the chromatography of a mixture of acid, basic and neutral compounds on a cyano column without the use of silanol suppressing agents. A cyano column was preferred as it can be used in both normal and reversed phase conditions. They found that the peak shapes of bases were improved as the ionic strength of the buffer was increased. According to the authors this is probably because the phosphate buffer masks the residual silanols.

Badiru and Jefferies, (1988) studied the chromatography of 16 drugs of abuse (see Results and Discussion) on low carbon loading columns i.e., cyanopropyl silica and trimethyl silica columns. They found that these columns, especially the CPS column, give short retention times with good resolution using predominantly aqueous mobile phases and were therefore suitable for use with post-column ion-pair extraction detectors as well as detection at low U.V (205 nm). Using low ionic strength buffers i.e 0.025M phosphate buffer, the peak shapes were not perfectly symmetrical, but were acceptable (A_S 1.0-1.3). Various other workers including Lawrence et al., (1979 a), Reddingus et al., (1981) Derendorf and Garrett, (1983) have all used cyano columns for the chromatography of tertiary amines with low percentages of organic modifier in combination with post-column ion-pair extraction detectors with DAS as the counter-ion.

As the present work is the continuation of the work reported earlier by Badiru (1989), a cyano column was therefore the starting point. As mentioned earlier Badiru and Jefferies, (1988) investigated the chromatography of drugs of abuse on cyano column at 205 nm. The purpose of the work was to use it in combination with post-column ion-pair extraction detection with naphthalene-2-sulphonic acid as a post-column ion-pairing agent (Badiru, 1989). The post-column system was needed to improve the selectivity of the method. However apart from other problems (see section 3.2) the sensitivity of the post-column system was less than that obtained at 205 nm.

In this work the chromatography of drugs of abuse was investigated on various columns so that at a later stage it could be used in combination with post-column ion-pair

extraction detector with DAS as counter ion. Fig 4.1 and 4.2 gives the structures of the drugs of abuse studied. The compounds codes are the same as those used by Badiru, (1989). TABLE 4.2 gives the important physico-chemical properties of these compounds.

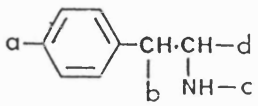
4.3 Results and Discussion

Cyano columns are intermediate polarity phases and can be used both for normal as well as reversed phase chromatography (De Smet et al., 1984). Under normal phase conditions the retention involves a competition between solute and solvent molecules for sites on the adsorbent surface. Synder (1983), Cooper and Smith (1986) & Smith and Cooper (1987) have studied retention on cyano bonded silica phases under normal phase conditions and have tried to describe retention in terms of an adsorption-displacement model. Salotto, (1990) compared the retention data for several solutes on diol, cyano and amino columns and found that cyano columns showed increased retention for dipolar compounds when compared to basic compounds.

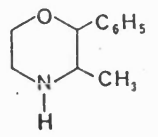
For reversed phase conditions the cyano bonded phases are marketed as an alternative to short alkyl chains with greater selectivity (Major,1980). DeSmet and Massart (1987) found that the retention behaviour on cyano columns is similar to other reversed phase columns i.e solute retention is a result of hydrophobic interaction and interaction with residual silanols.

Murthy et.al. (1991) studied five different brands of alkyl-bonded cyano materials used as sorbents for solid phase extraction cartridges. They found that the retention of basic drugs was achieved by a combination of hydrophobic, hydrophilic and ion-exchange mechanisms. 'The relative contribution of these mechanisms is affected by the relative population of cyano-propyl and end-capping silanes, the presence of residual silanols and other ionizable groups'.

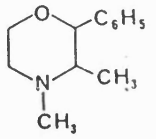
GROUP A SOLUTES



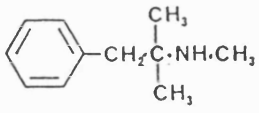
COMPOUND	CODE	a	b	c	d
p-Hydroxynorephedrine	1	OH	OH	H	CH_3
p-Hydroxyamphetamine	2	OH	H	H	CH_3
2- Phenylethylamine	3	H	H	H	H
p- Hydroxymethylamphetamine	4	OH	H	CH_3	CH_3
Amphetamine	5	H	H	H	CH_3
Methylamphetamine	7	H	H	CH_3	CH_3



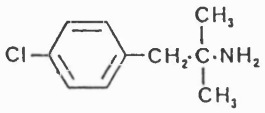
Phenmetrazine 6



Phendimetrazine 9



Mephentermine 8



Chlorphentermine 10

Fig 4.1 Structures of group A drugs studied

GROUP B SOLUTES

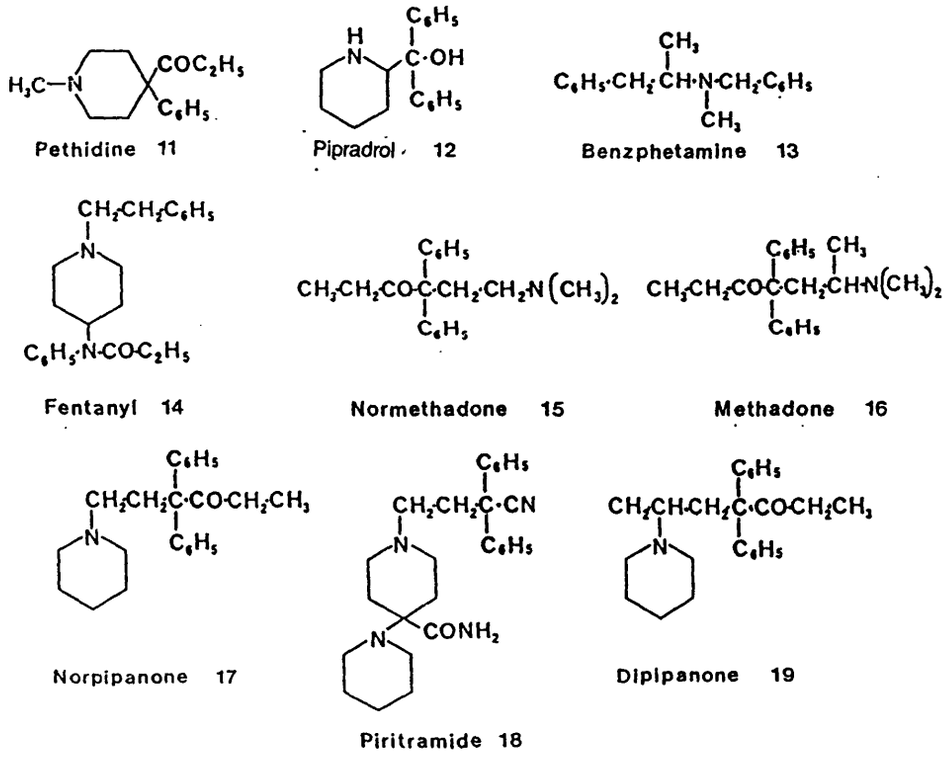


Fig 4.2 Structures of group B drugs studied

TABLE 4.2

Physico-chemical properties of analytical interest

Compounds	use**	log P *	pKa *	$A^{1\%}_{1cm}$ **
p-OH norephidrine (1)	sympathomimi-metic	0.18	8.6	110
p-OH amphetamine (2)	"	0.20	9.3	203
Phenylethylamine (3)		0.22	9.83	15
p-OH methyl-amphetamine (4)		0.22	–	–
Dexamphetamine (5)	stimulant	0.63	9.90	14
Phenmetrazine (6)	anoretic	1.02	8.40	13.3
Methylamphetamine (7)	central stimulant	1.69	10.10	12.1
Mephentermine (8)	sympathomi-metic	0.82	10.40	10.0
Pethidine (11)	Narcotic analgesic	0.72	8.7	8.5
Pipradrol (12)	central stimulant	0.35	9.71	13.4
Benzphetamine (13)	anoretic	0.54	6.66	19.1
Fentanyl (14)	Narcotic analgesic	1.51	7.34	13.0
Normethadone (15)	""	1.41	8.25-9.64	19
Methadone (16)	do	1.98	9.2	18
Norpipanone (17)	do	2.76	8.5	17
Piritramide (18)	do	2.15	8.5	
Dipipanone (19)	do	2.94	8.5-9.08	15.64

As salts all these compounds are soluble in water and ethanol, some are soluble in chloroform. They are generally insoluble in ether.

* Badiru, 1989

** Clark's Isolation and Identification of Drugs (1976)

It is generally agreed (Antle et. al. 1985, and Cooper and Lin, 1986) that the order of polarity of reversed phase columns is C_8 < phenyl < cyano. Initial work was carried out using a Spheri-5 cyano (Brownlee) column under the conditions described by Badiru and Jefferies for group B compounds, but problems were experienced regarding the stability of the column. Fig 4.3 (a) (b) shows a chromatogram of some Group B compounds at the beginning of the work (a) and one month later (b). This lack of stability was probably due to hydrolysis of the cyano-propyl chain from the bonding surface.

An attempt was therefore made to find a suitable stationary phase, probably a cyano bonded phase, which would be stable under the chromatographic conditions and give symmetrical peaks. Most of the commercially available cyano bonded phases are prepared by the chemical bonding of the silanol groups with alkyl propyl nitrile groups. However, coating of silica can also be achieved without making use of silanol groups for chemical anchoring. This is normally carried out by cross-linking and/or chemical bonding of special oligomers on modified or pretreated silica (presilanized or precapping) (Schomberg et al., 1983). This shielding of residual silanols by polymeric coating is more effective than silanized reversed phase materials and therefore basic compounds should elute as symmetrical peaks. According to Figge, (1986) the sample capacity and efficiency of polymeric coated columns is comparable to bonded columns. They are more stable with respect to loss of stationary phases especially with polar bonded phases e.g cyano phase with highly polar or extremely non-polar phases and give symmetrical peaks for basic compounds as compared to traditional phases.

Methyl-cyano, an 'evaluation column' from Phase Sep (Deeside, U.K) is a polymeric coated column with 10% carbon loading. The column was probably prepared using cyanomethyl polysiloxane XE 60 on an S5 silica (Figge, 1986). As this column was thought to be theoretically more stable than traditional columns and more suitable for basic compounds the chromatography of drugs of abuse was examined on this column

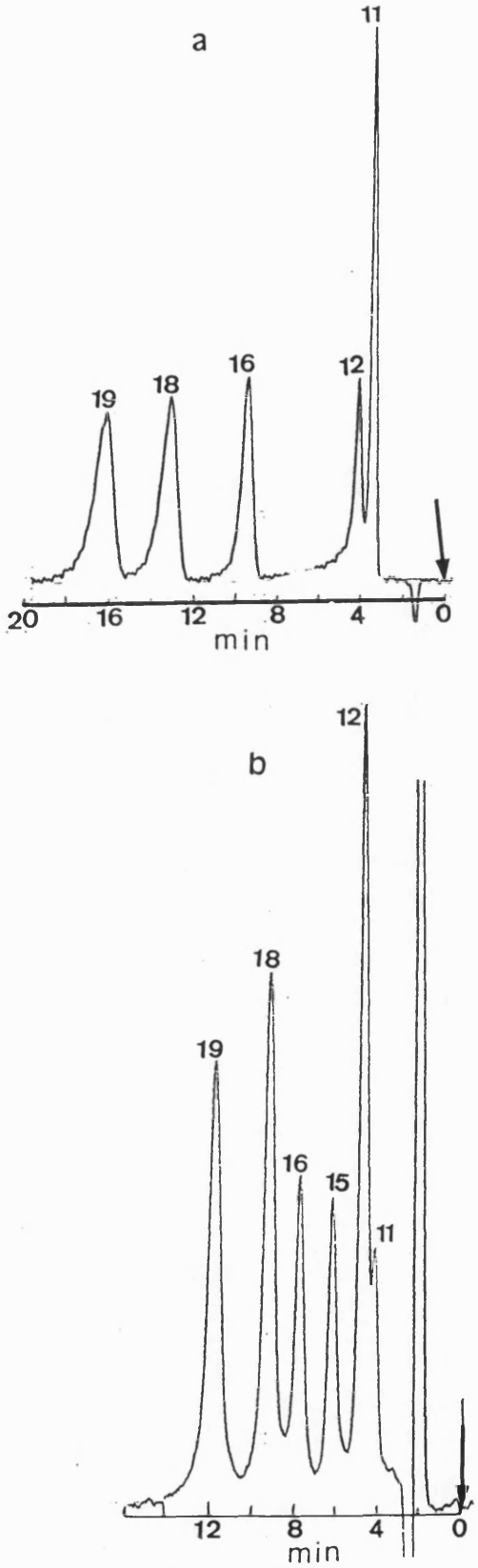


Fig 4.3 Sample chromatogram of some drugs of abuse on Spheri-5 cyano (a) post-column detection and (b) one month later, at 205 nm.

under the same conditions as specified by Badiru and Jefferies, 1988. and the results compared with Spheri-5 cyano (Brownlee).

4.3.1 Comparison of cyano propyl silica and methyl cyano phases

As can be seen from Fig 4.4 (a) analyte retention increased for all compounds on both columns as the pH of the mobile phase was increased. However the effect was more pronounced with the 'Spheri-5 cyano' (Brownlee) column than with methyl-cyano column. At pH 7.2 'Spheri-5 cyano' shows greater retention for all compounds than methyl-cyano, whereas at pH 4.0 the methyl-cyano shows greater retention for all the compounds tested. The behaviour of these two different phases can probably be explained by the presence of residual silanol groups. It is probable that the Spheri-5 cyano has more residual silanol groups than the methyl cyano, and at pH 7.0 as more silanol groups are ionised, the percentage protonation of bases is reduced and the stationary phase is preferred. The analyte retention caused by the hydrophobic interaction with the bonded surface is also increased. The methyl cyano has less residual silanol groups and the retention of compounds is mainly due to their hydrophobicity. This is also confirmed by the fact that the retention order of compounds 1-8 is related to their log P values. The relationship was not linear however as can be seen from TABLE 4.3

TABLE 4.3 Relationship of log k' vs log P for group A compounds

Compound (code)	log P	log k'.
1	0.18	0.27
2	0.20	0.36
3	0.22	0.50
4	0.22	0.50
5	0.63	0.58
6	1.02	0.72
7	1.69	0.86
8	0.82	0.79

r=0.880, intercept 0.363 and slope 0.334.

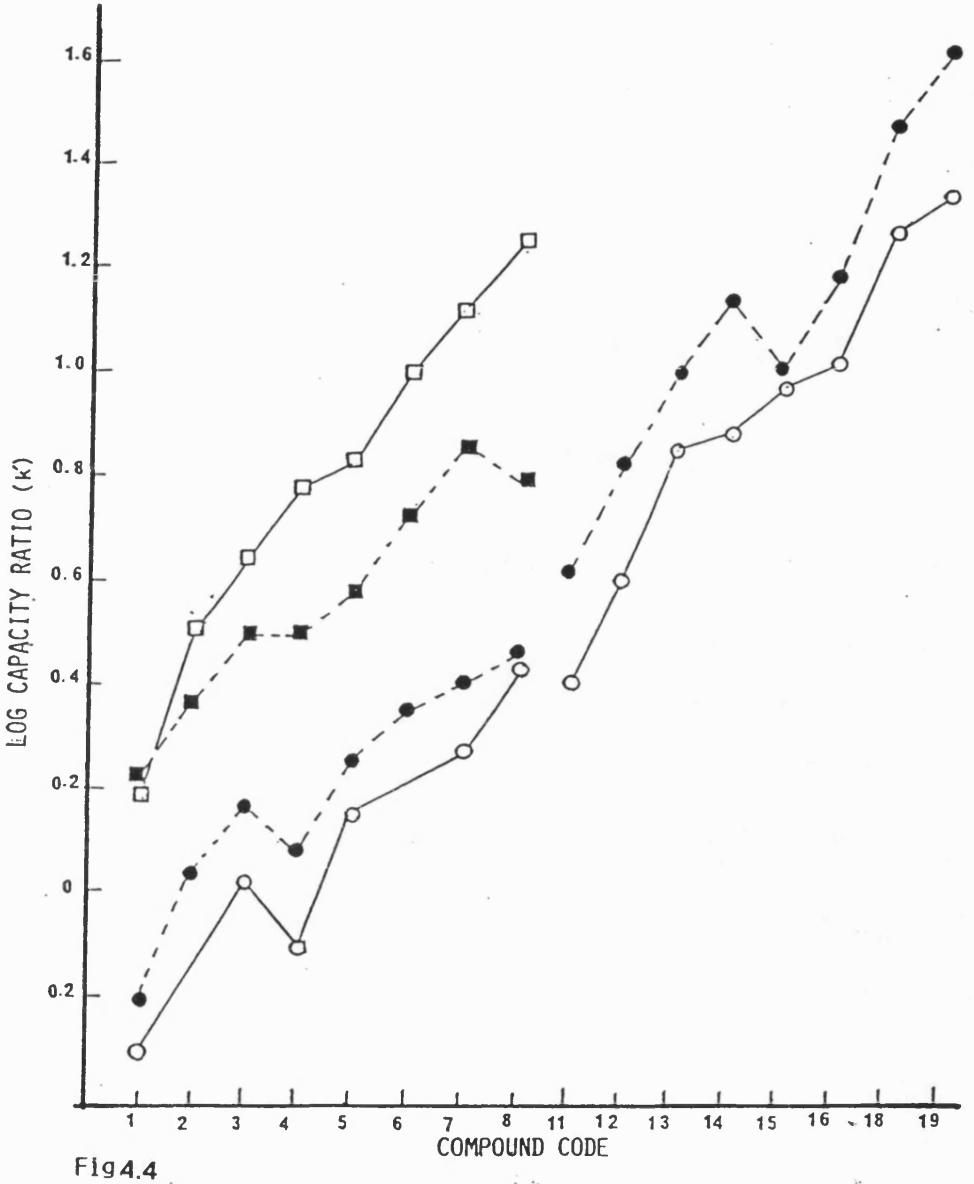


Fig 4.4

(——) Brownlee Spheri-5 Cyano (100 x 2.1mm I.D)

(----) Methyl/cyano polymer coat 10% loading on S5W Silica (100 x 2.0mm I.D.) - phase SEP

●○ pH 4.00 (A)

■□ pH 7.20 (B)

(A) 0.025M phosphate buffer-propan-2ol-acetonitrile (76 : 12 : 12)

(B) 0.025M phosphate buffer-methanol-acetonitrile (90 : 5 : 5 v/v/v)

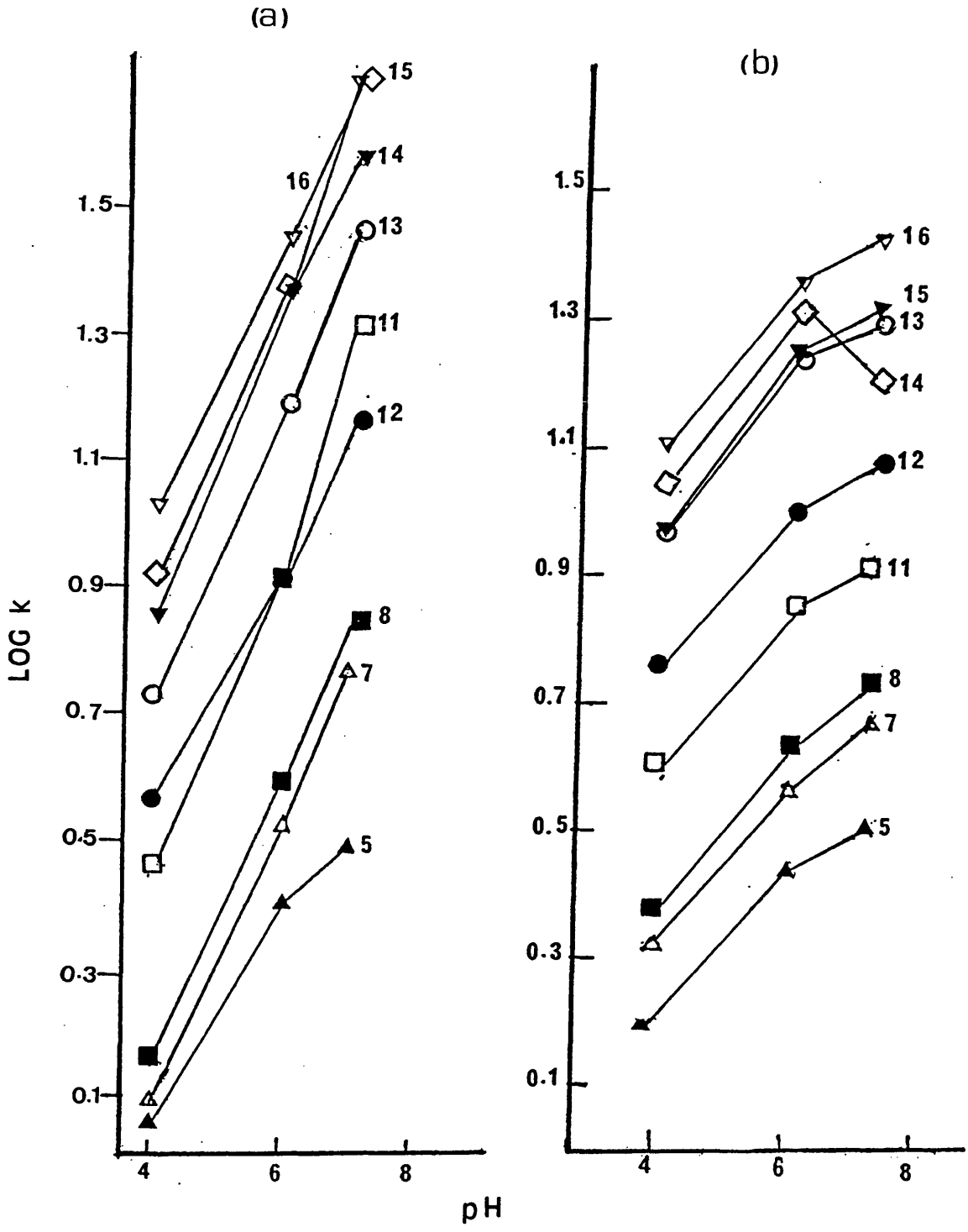


Fig 4.5 Effect of pH vs log k' (a) Spheri-5 cyano (b) Methylcyano

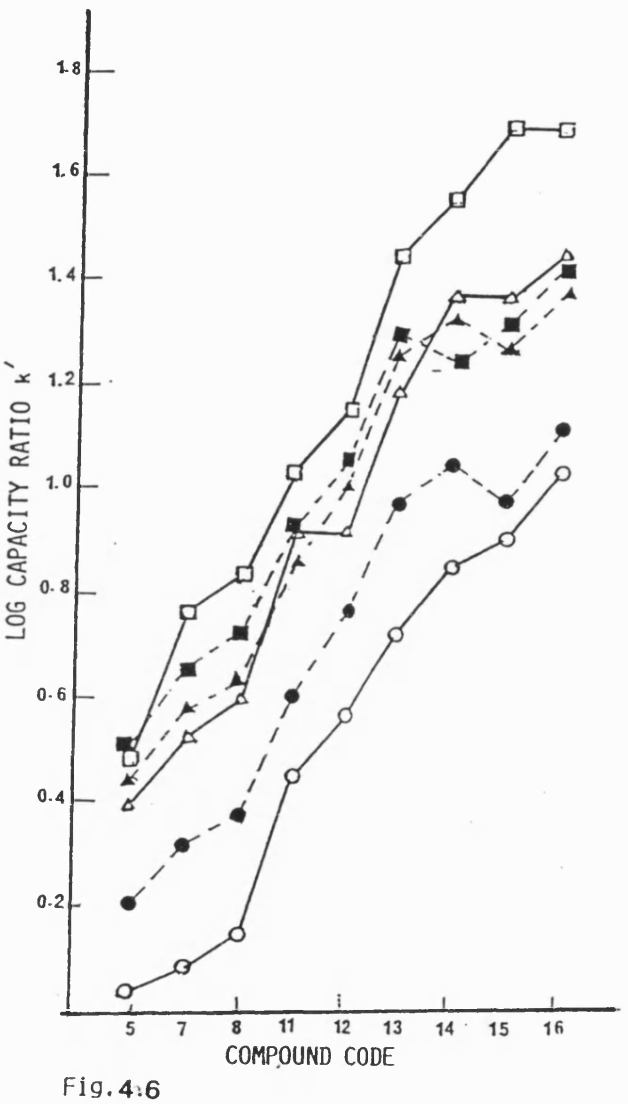


Fig.4.6

(——) Brownlee Spheri-5-Cyano (100 x 2.1mm I.D) Column
(----) Methyl Cyano Polymer Coat - Phase Sep
● ○ pH 4.00
▲ △ pH 6.00 mobile phase A as Fig.
■ □ pH 7.20

At the optimum pH (4.0) for Group B compounds (11-19), Group A compounds eluted very rapidly. At this pH the ionization of silanols is mainly suppressed, the bases are more protonated and the mobile phase is preferred. Under these conditions the methyl cyano shows greater retention than Spheri-5 cyano due to the higher carbon loading.

To further confirm the effect of pH a representative sample of both analyte groups was used consisting of Compounds 5, 7, 8, 11, 12, 13, 14, 15, The pH of the mobile phase [consisting of 0.025 M phosphate buffer - propan-2-ol - acetonitrile (76:12:12 v/v/v)] was varied from 4.0 to 7.0 and log κ' was plotted vs pH. (Fig 4.5 (a) and (b)). A comparison of Fig 4.5 (a) and (b) show that the slopes of log k' vs pH are much steeper on Spheri-5 cyano than on the methyl cyano, which is the anticipated result if the percentage of residual silanol groups on the Spheri-5 cyano material has a greater influence. At pH 6.0 the effect of pH is intermediate on both columns so that compounds 5,7,8,12,13 show greater retention, and 11,14,15,16, show less retention on methyl cyano as compared to Spheri-5 cyano, Fig 4.6. Under the conditions used, it was not possible to find a simple explanation for this behaviour as it was not directly related to either the pKa (6.6 to 10.4 for first set and 7.34- 9.20 for the second set) or log P values of the compounds. A combination of pKa, log P and other factors may be involved.

4.3.2 Prediction of retention in reversed phase chromatography

A. Partition coefficients

In developing a chromatographic separation for pharmacologically active substances e.g drugs of abuse and their metabolites, it would be an advantage if eluting conditions could be predicted from the information which is available e.g nature of the column, eluting strength of the mobile phase and the structure of the analyte, so that a minimum number of experiments would be needed to obtain the optimum chromatographic conditions for the required separation.

Under ideal conditions the retention on alkyl bonded silica should depend on the solute molecules interacting with the alkyl groups as a result of london-dispersion forces (Colin

et al. 1983). The interaction of alkyl chains with the solute molecules depends upon the length of alkyl chain and the composition of the mobile phase.

However as mentioned earlier, other interactions apart from hydrophobic interaction can also occur e.g interaction of solute molecules with silanol groups, this effect is more pronounced with polar and basic solutes. Various methods have been proposed to predict retention in RP-HPLC e.g De Smet and Massart 1987 tried to relate log k' to carbon number (Cn) for acidic, basic and neutral drugs. According to the authors a linear relationship should exist between log k' vs Cn

$$\log k' = a + b\,Cn \qquad \text{Eq 4.1}$$

Another approach is the use of partition coefficients. The partition coefficients have a linear relationship with log k' measured under reversed phase conditions for neutral molecules (retention of a solute under unionised form) (Thus and Kraak, 1985).

$$\log k' = y \log P + m \qquad \text{Eq 4.2}$$

where y and m are the slope and intercept of the least squares regression line.

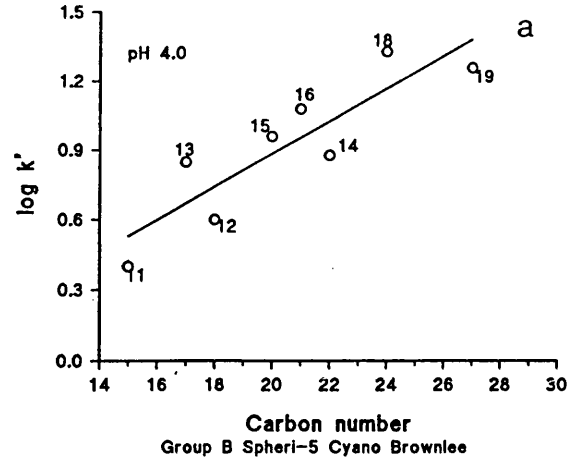
Fig 4.7 (a) and (b) show the plot of log k' vs Cn on Spheri cyano and methyl cyano columns. Fig 4.8 shows a plot of log k' vs log P on both cyano columns. The results are shown in TABLE 4.4

TABLE 4.4

	Column	r	slope and intercept y=
Cn	Spheri-cyano	0.882	0.0712(x) -0.504
	Methyl-cyano	0.899	0.073(x) -0.31
	methyl cyano (excluding 17,19)	0.981	0.071 (x) -0.415
log P	Spheri-cyano	0.939	0.260(x) + 0.589
	Methyl-cyano	0.927	0.275 (x) + 0.757

For Group A compounds the correlation between log k' vs Cn or log P was very poor , as can be seen from Fig 4.9 (log k' vs log P)

The relationship between log k' vs Cn or log P for neutral or non polar compounds should be linear as the retention mechanism is mainly hydrophobic interaction. However for polar or basic compounds where silanophilic interactions predominate the relationship is



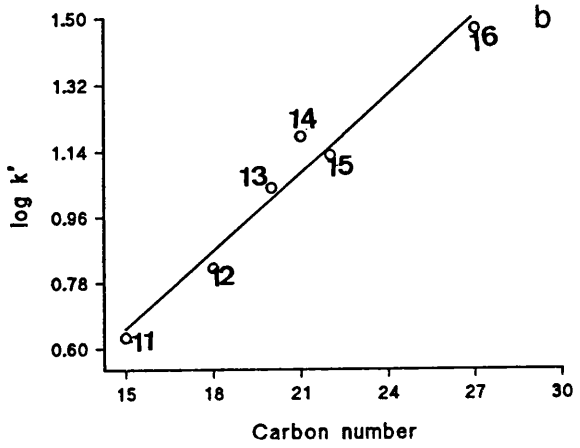


Fig 4.7 A plot of carbon number vs log k' for group B compounds (a) Speri-5 cyano (b) methylcyano columns

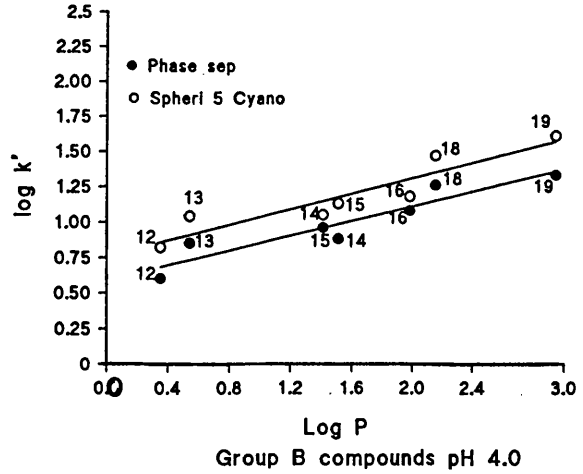


Fig 4.8 A plot of log P vs log k' for group B compounds on both columns.

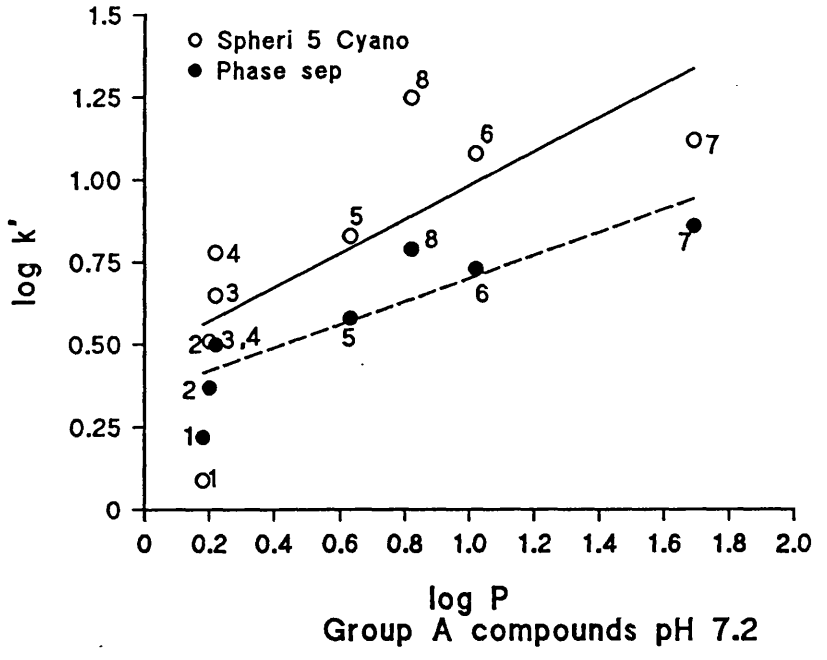


Fig 4.9 A plot of log P vs log k' for group A compounds on both columns

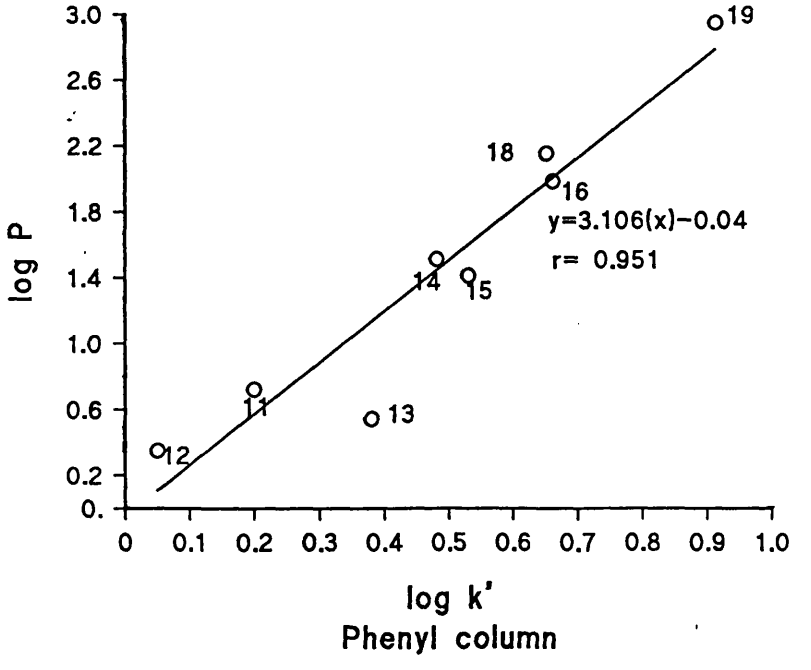


Fig 4.10 A plot of log P vs log k' for group B compounds on a phenyl column

not linear because in this equation there is no factor which takes into account the silanophilic interactions.

To obtain a better correlation between log k' and log P for ionizable substances various other approaches have been used. For example Unger and Chiang, (1981) obtained a correlation of 0.996 when NN dimethyloctylamine (DMOA) was added to a buffer of pH 7.2. Without DMOA the correlation was 0.867. The DMOA masks the residual silanols. To remove silanophilic interactions, De Biasi et al., (1986) chromatographed bases at pH 13.4 (bases are unionised at this pH) on a polymeric column, and obtained a correlation of 0.906. Kraak et al., 1986 used a phenyl bonded column with an ion-pairing agent (Sodium dodecyl sulphonate). According to the authors the phenyl bonded phase seemed a better stationary phase than ODS for obtaining log P values from retention data or vice versa, and it also gave shorter retention times.

Fig 4.10 shows a plot of log k' vs log P for Group B compounds on a phenyl column using the same mobile phase as for the Spheri-5-cyano column. Under the conditions used, the phenyl column shows a better correlation between log k' values and log P than spheri-cyano or methyl cyano columns. Pietrogrande et al., (1987) also found that a phenyl column was best for the prediction of log P values for a series of benzodiazepams, while cyano columns were sensitive to functional groups (OH). This may be due to better masking of residual silanols by the phenyl moieties and the possibility of π–π interaction between the analytes and the phenyl moieties (Thus and Kraak 1985).

B. Prediction of retention time from dissociation constants

The retention time of the unionized form of a compound can be predicted by the relationship between log P vs log k' (Hanai, 1991). For ionisable substances if the retention times for the molecular form (unionised) and the ionic form are known, then it would be possible to predict their retention time at a given pH (Hanai et al., 1982).

However under reversed phase conditions with alkyl bonded silica it is not possible to obtain retention times for the molecular form of strong bases because of the inability of these phases to withstand a high pH. Hafkenshied and Tomlinson (1984) in their work have used an equation (given below) which can be used to obtain retention data for the molecular form of the base utilizing the data obtained at a pH which can be easily used on such columns.

$$k' = [1+10^{(pH-pKa)mob}]^{-1} k^0 + [1+10^{(pH-pKa)mob}]^{-1} k^+ X\ k^+ . \qquad \text{Eq 4.3}$$

where k is the non logarithmic capacity factor of the chromatographed species be it solute (k'), its uncharged form (k^0) or its monoprotic charged form (k^+), pKa is the solute pKa under mobile phase conditions and pH is the mobile phase pH.

Using the pKa values determined by Badiru, (1989), the above equation was used to calculate k^0, and consequently predict k' at different pHs. Fig 4.11 and 4.12 show the relationship between predicted log k' and experimental log k' at pH 6.0 on both the columns. Excellent correlations were obtained for Spheri-5-cyano (r=0.993) and methyl cyano r=0.998, although with negative intercepts. It seems from these results that some other factors are also responsible for retention of the analytes which this equation is not taking into account e.g silanophilic interactions.

Hanai 1991 used a similar equation for prediction of retention times

$$k' = A\ \frac{k_m - k_i}{2} \tan h\ (pKa - pH) + \frac{k_m + k_i}{2} \qquad \text{Eq.4.5}$$

where k_m is the capacity factor of the molecular form (unionised form), k_i is the capacity factor for the fully ionised form i.e zero, A is a constant, whose value can be adjusted to improve the agreement between predicted values and the chromatographic data.

If the negative intercepts are the result of silanophilic interactions then the use of a polymeric column may provide improved predictions.

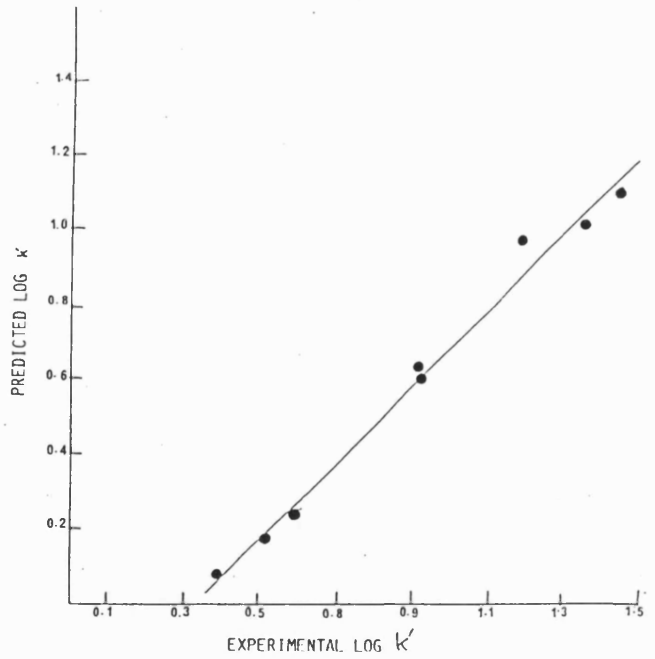


Fig 4.11
Prediction of log k' at pH 6.0 mobile phase (A) as in Fig 4.4 using Hafkensheid & Tomlinson equation.

Brownlee Spheri-5 cyano (100 X 2.1 mm i.d)
r=0.993 (n=8) intercept = -0.332, slope = 1.02

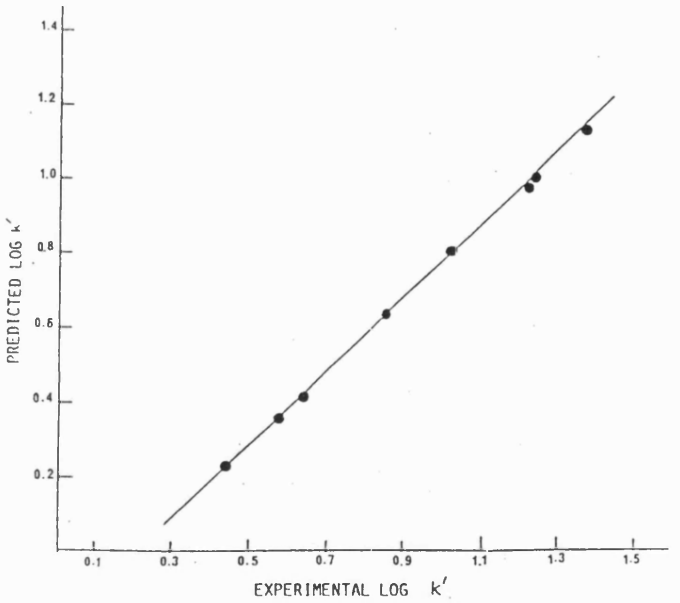


Fig 4.12
Prediction of log k' at pH 6.0 mobile phase (A) as in Fig 4.4 using Hafkensheid & Tomlinson equation.

Methyl/cyano polymer coat (10 % Carbon loading on S5W Silica) (100 X 2.1 mm i.d) Phase Sep.
r=0.998 (n=8) intercept = - 0.209, slope = 0.985

4.3.4 Evaluation of cyano columns using selected solutes

Smith and Miller (1989) used retention indices based on an alkyl aryl ketone index and examined various commercial cyano bonded phases using a series of test solutes. They found considerable differences among various suppliers. Although the use of retention indices is desirable in order to minimise problems associated with the measurement of t_0 and also because retention indices are less affected by small changes in mobile phase composition than capacity factors, they were not used in this work due to the short lives of the columns and k' values were used instead.

The test solutes used were nitrobenzene (NB), toluene (T), 2-phenylethanol (2-PE), p-cresol (p-C), methyl benzoate (MB) N-methyl aniline (NMA) [pKa 4.85] and N-N-diethylaniline (NNDA) [pKa 6.57]. The first four compounds were used to study for different interactions that can occur on a column (Smith, 1984 a). N-methyl aniline and N-N-diethylaniline to account for silanol interactions, methylbenzoate has been used as a secondary standard on an ODS column (Smith, 1984 a). The solutes were chromatographed using methanol-0.025M phosphate buffer at pH 7.0 and at pH 4.0. The results of Spheri-5-cyano and methyl cyano were compared with Hypersil CPS and are shown in TABLE 4.5.

TABLE 4.5

Solutes	Spheri-5-cyano κ'		Methyl cyano κ'		Hypersil CPS κ'	
	pH 7.0	pH 4.0	pH 7.0	pH 4.0	pH 7.0	pH 4.0
NMA	1.95	1.52	1.74	1.53	0.713	0.65
2PE	1.57	1.64	1.15	1.20	0.51	0.59
T	3.82	3.98	2.95	3.09	1.19	1.23
NB	2.87	2.94	2.25	2.35	0.90	0.94
MB	3.98	4.07	2.48	2.59	1.196	1.25
p-C	2.05	2.12	1.60	1.66	0.69	0.70
NNDA	12.22	3.48	7.23	3.23	4.14	1.52
MB/T	1.02	1.02	0.84	0.83	1.0	1.0

As can be seen from TABLE 4.5 for basic analytes as the pH is lowered from 7.0 to 4.0 retention is reduced. If the results of all the columns shown in TABLE 4.5 are compared it seems that the Spheri-5-cyano has the most silanol interaction and most hydrophobicity and the Hypersil CPS has the least silanol interactions. The order of elution of MB and toluene is the same for Hypersil CPS and Spheri-5-cyano but is reversed for methyl-cyano columns. According to Engelhardt and Jungheim (1990) for polymeric phases where the silanols are better shielded, the ester always elutes before toluene whereas for monomeric phases the ester elutes after toluene. The data for the methyl-cyano column confirms this test.

TABLE 4.6

Solutes	Spheri-5-cyano κ'^*		Methyl cyano κ'^*		Hypersil CPS κ'^*	
	pH 7.0	pH 4.0	pH 7.0	pH 4.0	pH 7.0	pH 4.0
NMA	1.24	0.92	1.51	1.27	1.39	1.10
2PE	1.0	1.0	1.0	1.0	1.0	1.0
T	2.43	2.58	2.56	2.56	2.33	2.08
NB	1.82	1.79	1.95	1.95	1.76	1.59
MB	2.53	2.48	2.15	2.15	2.34	2.11
pC	1.30	1.29	1.39	1.39	1.35	1.18
NNDA	7.78	2.12	6.28	2.69	8.11	2.57

* normalised κ' values

The data for TABLE 4.6 was obtained from TABLE 4.5 by dividing the capacity values of all the test solutes with that of phenylethanol. This was done to exclude any variations in the results.

The results for all the test solutes are very similar on all the columns (the difference is not greater than 0.15) except NNDA where Hypersil CPS show much more silanol activity as compared to methyl cyano. Theoretically the methyl-cyano should show least silanol activity when compared to other columns because of better shielding of silanols by the polymeric layer.

Spheri-5-cyano and methyl-cyano under the conditions used had very short lives, moreover the manufacturer had problems in producing reproducible polymeric coated cyano columns so further work with these columns was not pursued. For the chromatography of drugs of abuse a Spherisorb-5 cyano SGE column was therefore used.

Engelhardt and Jungheim 1990 have suggested more pragmatic tests for the characterization of stationary phases using selected solutes. According to the authors using standard conditions i.e methanol-water 55-45% v/v it is possible to distinguish between RP8 and RP18 using toluene and ethylbenzoate. With RP 8, ethylbenzoate is always eluted together or after toluene whereas with RP18 ethylbenzoate elutes before toluene. 'Good columns' for bases can be checked by looking at the elution pattern of aniline and phenol. For 'good' phases aniline always elutes before phenol and the peak asymmetry relationship of the aniline/phenol peaks is less than 1.3. The test can also be used to check column stability towards hydrolysis.

The columns listed in TABLE 2.1 were tested using methanol-water, and only pKb-100 and SGE C_8 passed the test for 'good columns' for bases. Hypersil BDS passed the 'aniline phenol' test initially but on storage in 50% methanol water for a month failed the 'aniline phenol' test, but gave excellent peak shapes for neutral solutes (see Chapter 6).

5 COCAINE & METABOLITES

5.1 Introduction

Cocaine is one of the most widely used drugs of abuse. A survey carried out in 1986 in America (Isenschmid et al., 1988) showed that 4-6 million Americans use cocaine, approximately 200,000 are addicts and about 5000 people will try the drug each day. A more recent survey carried out in America (Hearn et.al., 1991) indicate that over 12 million people concurrently use cocaine with ethanol. The situation in other parts of the world may not be as alarming as that but there is concern by Government authorities to prevent a cocaine epidemic in the U.K. Evidence for this threat from cocaine is the large number of cocaine seizures by Customs and the Police which are frequently reported in the media. It is unlikely that cocaine abuse can be prevented, however, and it is important to develop sensitive and specific analytical methods for its detection in order to enforce the law and to permit improved pharmacokinetic studies to be carried out.

Cocaine (Farrar and Kearns, 1989) is one of the 14 alkaloids found in the leaves of "*Erythoxylon coca*" which is widely cultivated throughout South America in the foothills of the Andes and in the Amazon regions. The plant leaves contain 0.5% w/w of the alkaloid. Cocaine paste, which is extracted from these leaves contains about 80 % of cocaine. The alkaloid is usually converted to the hydrochloride salt which is then diluted with talc, arsenic, sugar or lidocaine and sold for nasal insufflation (snorting) or I.V use. The purity of street cocaine varies between 20-80%. Free base cocaine or "crack" is the salt dissolved in alkaline solution and recrystallised from ether. It has a lower Mpt than the salt and is thus ideal for smoking or "free basing".

5.2.History

Cocaine was introduced to Europe as coca-leaf in 16th century. Albert Niemann isolated cocaine from coca leaf in 1860. In 1923 Richard Willstatter determined its structure and synthesized it. Carl Koller reported its value as a local anaesthetic for which it is still used e.g in endoscopy and ophthalmology.

At the beginning of this century cocaine enjoyed popularity as a component of stimulants like tonics, sodas and Coca-cola. In 1903 cocaine was excluded from Coca-cola and was banned in 1914 by the Harrison Act in the U.S.A. (Jatlow 1989).

5.3. Preparations of cocaine and cocaine HCl (Martindale 29th Ed)

Cocaine eye drops BPC 1973.

Cocaine eye drops strong APF.

Cocaine & Homatropine eye drops BPC 1973.

Cocaine HCl tablets for topical solution USP.

Cocaine & Adrenalin paste 10% APF.

Cocaine & Adrenalin paste 25% APF.

Cocaine may be used topically in solutions of 1-20% although concentrations not greater than 4% are recommended to reduce incidence of side effects. Solutions for ophthalmological use generally contain up to 4 % and 0.25 - 0.5% are used as a corneal anaesthetic.

Street names

Bernice, blow, C, Charlie, coke, crack, flake, girl, gold dust, her, lady, leaf, nose, candy, rock, she, snow, toot, white lady, white girl (Martindale 29th Ed.).

5.4. Pharmacology

Although the pharmaceutical preparations of cocaine exploit its properties as a local anaesthetic, it is also a powerful central nervous system stimulant. The stimulant effects of cocaine produce an elevation of mood and euphoria which causes an overwhelming desire to continue taking the drug, resulting in psychological dependence (Martindale 29th ed). It is considered to be the most reinforcing of all the psychoactive drugs. Animals given free access to the drug will self administer it to the point of death (Jatlow, 1989). Repeated use may result in acute tolerance to some effects. e.g cardiovascular and behavioural effects. Abrupt cessation may result in withdrawal symptoms which include fatigue, lassitude, depression and irritability.

Cocaine abuse may give rise to psychotic states and hallucinations, nausea, vomiting, loss of appetite, weight loss, formication (so called cocaine bugs), decreased fatigue, rapid pulse, increased temperature, and rapid shallow respiration. Convulsions, coma and death from respiratory failure may occur. Prolonged cocaine abuse by nasal inhalation, "snorting" results in mucosal damage and perforation of the nasal septum. Cocaine is sometimes injected with diamorphine to control toxic symptoms, (Martindale 29th Ed) the so-called combination of "high and low". Cocaine use in patients with coronary heart disease causes acute myocardial infarction.

As cocaine causes placental vasoconstriction, in pregnant women there is an increased risk of spontaneous abortion. Infants exposed to cocaine through maternal use are at risk of congenital malformations, prenatal mortality and neurobehavioural impairments (Roe et.al, 1990). The harmful effects of street cocaine may be a lot worse because of the presence of other harmful substances as mentioned earlier.

5.5. Kinetics & Metabolism

Historically, the habit of chewing coca leaves is still a major route of administration among the inhabitants of Peru, who consume vast quantities of coca leaves each year (Ritchie and Greene, 1990). In a survey carried out in USA in 1981 (Poklis et al., 1985), among addicts, it was found that 61% were intranasal users, 21% free base smokers, and 18% intravenous users. Absorption is good from all sites of administration especially if there is inflammation (Martindale). With oral administration there is a lag phase of about 30 min. before maximum concentrations are reached in 60 min (Jatlow, 1989) because cocaine is absorbed from the lower gastrointestinal tract. The nasal route is popular among addicts for recreational administration. Maximum concentrations are not reached for 30-60 min, probably because of local vasoconstriction. Systemic bioavailability is 20-60 % as compared to intravenous (Jatlow, 1989). Smoking and the intravenous route both give instantaneous access to the systemic circulation and brain. The lung provides a large surface area for absorption. Smoking free base cocaine is more efficient than smoking paste.

Cocaine is very lipophilic and concentrations in the CNS are four times higher than in plasma (Farrar and Kearns, 1989). It has also been found that cocaine can be detected for 5 to 10 days (Cone and Weddington, 1989) after chronic use of cocaine, which suggests that cocaine may accumulate in some tissues and then be slowly released into the circulation.

Cocaine is widely distributed within the body and Poklis (1985) found that in a fatal case after a high IV dose, cocaine concentrations in mg/kg were : kidney 26, spleen 22, brain 14, heart 6.1, skeletal muscles 6.1, lungs 3.4, liver 1.6, and adipose issue 1.0. The distribution in body fluids (mg L^{-1}) was shown by the same study to be : urine 39, bile 10, vitreous humour 2.4, and blood 1.8.

Approximately only 1.5% of cocaine dose is excreted unchanged in the urine. The bicyclic structure of cocaine is characterised by functional groups including N-methyl, carboxymethyl ester and benzoyl ester, all of which are susceptible to biotransformation, as shown in Figure 5.1.

For a given dose of cocaine, 46% is excreted as benzoylecgonine (BE), 41% as ecgonine methyl ester (EME), 3% as cocaine and 10% as demethylated (i.e., norcocaine) hydroxylated products and ecgonine (Ambre 1985). According to Inaba, (1989) the esterases play a major role in cocaine metabolism. For example ecgonine methyl ester is produced by the action of plasma cholinesterase on the benzoyl group of cocaine (Stewart et al., 1977). N-demethylation of cocaine (mediated via cytochrome P-450) to produce norcocaine is higher in subjects with lower plasma cholinesterase activity. (Inaba et al.,1978) which indicates that norcocaine is also hydrolysed enzymatically (Stewart et.al 1979). According to the above authors benzoylecgonine is produced by non-enzymatic hydrolysis. Other minor metabolites which have been found include among others hydroxycocaine, methylecgonidine in the bile of cocaine users (Lowry et al., 1979), and arylhydroxy and hydroxymethoxy metabolites in the urine of polydrug users (Smith et al 1984). Ethylcocaine (Rafla and Epestein, 1979) and ethyl esters of arylhydroxy and arylhydroxymethoxy cocaines (Smith 1984 b, c) have been detected in persons who concurrently abuse cocaine with ethanol. Ethylcocaine is produced by transesterification in the liver, a reaction catalysed by liver homogenates (Roberts et al., 1992). The CNS effects of ethylcocaine are more potent than that of cocaine (Hearn et al., 1991). It has also been found that in acidic ethanol solutions cocaine is converted into ethylcocaine (Smith,1984 c).

According to Ambre, (1985) the relative proportions of cocaine, EME, and BE in the urine may be helpful in establishing the time elapsed since administration. As can be seen in Figure 5.2. (Ambre, 1985), the curves indicate the relative amounts of cocaine, BE and EME after cocaine dose.

Fig 5.1 COCAINE METABOLITES

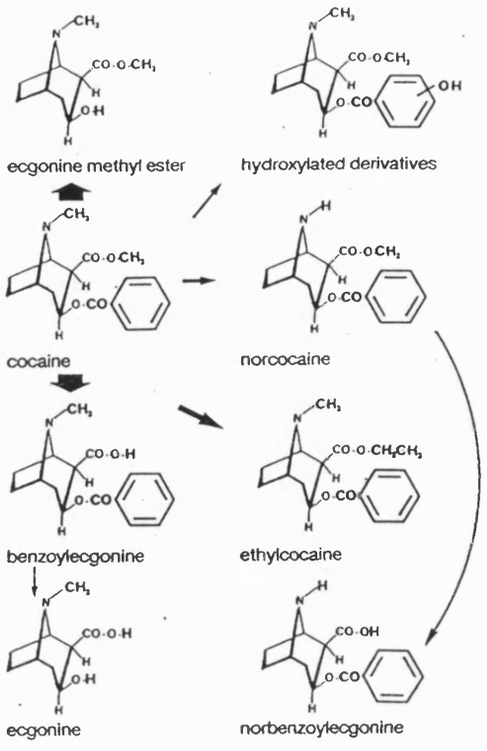
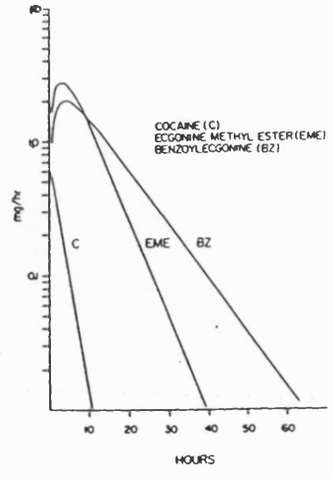


Fig 5.2 Excretion of cocaine, ecgonine methyl ester and benzoylecgonine

Semilog plot of excretion rate curves for urine following a single 100 mg IV dose of cocaine, from J Ambre, J.Analyt.Toxicol, 9, November/December 1985.



BE is slowly excreted in the urine and has a long half life of around 5-8 hrs in contrast to cocaine 0.5-1.5 hrs and EME 3.5-6.0 hrs (Jatlow, 1989). This has been the reason why most analytical methods for cocaine are based on the presence of benzoylecgonine in the urine. According to NIDA (National Institute of Drug Abuse) the method used for confirmation of cocaine abuse using urine samples should have a cut-off value of 300 ng ml^{-1} for BE (Ortuno et al 1990). The choice of analytical method for the detection of cocaine and its metabolites in urine should primarily be based on the detection of not only BE, but also EME and cocaine, as EME accounts for more than 41% of a given dose excreted in the urine. Although norcocaine, which is an active metabolite of cocaine and its metabolites have been suggested as mediator of hepatotoxicity in rats (Jatlow, 1989), the amount found in human urine is < 1% therefore this metabolite is not very important for analytical purposes. Ethylcocaine is an important metabolite as most cocaine users abuse alcohol, so that it is frequently present. It has much the same CNS effect as cocaine and is responsible for increasing the toxic overdose of cocaine.

The objective of this study was to develop a sensitive method for detection of cocaine and its major metabolites i.e., BE and EME which could not only meet the NIDA guidelines for BE, but also serve as a general screening method for detection of cocaine abuse.

5.6. Physicochemical properties

COCAINE HCl $C_{17}H_{21}NO_4$ HCl =339.8 (Clark)

Odourless hygroscopic colourless crystals or white crystalline powder. Mpt about 197° with decomposition. Cocaine hydrochloride 1.12 gm is approximately equivalent to 1 gm. of cocaine base.

Soluble 1 in 0.5 of water, 1 in 3.5 to 4.5 of alcohol, 1in 15-18 of chloroform, soluble in glycerol, practically insoluble in ether and fixed oil. Protect from moisture and light.pKa 8.6 (20^{o}). $A^{1\%}_{1cm}$= 450, λ max =233 nm.

BENZOYLECGONINE $C_{16}H_{19}NO_4$ = 289.3 (Clark)

The hydrated form occurs as crystals and melts at 86^{o}. The anhydrous form melts at 195^{o}, with decomposition. Very soluble in hot water, soluble in ethanol, practically insoluble in ether, soluble in dilute acids and alkalies. $A^{1\%}_{1cm}$= 375, λ max =233 nm.

ECGONINE $C_9H_{15}NO_3$ =185.2 (Clark)

Soluble in water, slightly soluble in ethanol, practically insoluble in ether, pKa 2.8 and 11.1 $A^{1\%}_{1cm}$= 50, λ max =233 nm.

ETHYLCOCAINE $C_{18}H_{32}NO_4$ = 317.37 (Merck Index)

Prism crystals from alcohol, almost insoluble in water, soluble in alcohol and ether.

Mpt. 109 ^{o} C. $A^{1\%}_{1cm}$= 430, λ max =233 nm.

5.7.Literature review of analytical methods

The analytical methods for the identification and quantification of cocaine and its metabolites can be classified as being either general screening methods or confirmatory techniques:

5.7.1 General screening methods

These include thin layer chromatography (TLC) and immunoassays. These methods are used as primary procedures to detect cocaine abuse in 'unknown' or suspect samples. If positive samples are found they are then checked by confirmatory techniques i.e GC-MS or HPLC.

TLC is normally carried out on silica plates with normal phase solvents. Metabolites are extracted from urine or other biological fluids and the extract is spotted on to the plates (Valanju et al., 1973). In certain cases the metabolites in the extract can be derivatized to

avoid interferences (Bastos et al., 1974). After development the plate is sprayed either with Dragendroff's reagent or acidified iodoplatinate reagent. The R_f values of the samples vs standard and the colour response to the reagent are used to identify the presence or absence of the drug or its metabolites. TLC is not very sensitive, around 3-5ug/ml for BE (Bastos et al 1974). A comparative study by Wallace et al. (1977) found that although the precision of TLC was poor compared to GC it was most applicable for determining large number of drugs simultaneously.

Immunoassays are more reliable and sensitive than TLC and include RIA (radio-immunoassay), EMIT (Enzyme multiplied immuoassay technique) and TDx (cocaine metabolite fluorescence polarization immunoassay FPIA) (Smith and Joseph, 1989).

Immunoassay techniques utilise antibodies specific for particular proteins or other large and small molecules (antigen). A competition occurs between the antigen to be quantified and the antigen attached to a chemical entity (label) which enables it to be detected by measurement either of radioactivity (RIA), fluorescence (TDx), or enzyme activity. The greater the quantity of antigen present in the sample the less labelled antigen will combine with the antibody.

Radio immunoassay requires :- a) an antibody which combines specifically and with high affinity with the substance to be assayed, b) a radioactive labelled version of the substance to be assayed, c) a method for separating antibody bound from serum material in solution either by gel filtration, addition of activated charcoal or alternatively the antibody is precipitated by the addition of suitable anti-IgG (Rang & Dale, 1987).

RIA is highly specific for BE with a cut-off level of 20 $ngml^{-1}$ for both blood and urine. RIA is not intended for the detection of unchanged cocaine, but can be used when properly standardised and in the absence of BE.

Enzyme immunoassay techniques (EMIT) use enzymes instead of radioactive material and thus avoid the problems associated with radioactive licensing or disposal of radioactive wastes. EMIT is also highly specific for BE. The assay is based on competition between the drug in the sample and the drug labelled with the enzyme glucose 6-phosphate dehydrogenase (G6P-DH) for antibody binding sites. Enzyme activity decreases upon binding to the antibody, so that the drug concentration in the sample can be measured in terms of enzyme activity. The active enzyme converts nicotinamide adenine dinucleotide (NAD) to NADH, resulting in an absorbance change which is measured spectrophotometrically (Emit d.a.u.™ Cocaine Metabolite Assay 1989 Syva Corporation). The low and medium calibrators contain 0.3 and 3.0 $\mu g\ L^{-1}$ of BE respectively. Ecgonine and cocaine are also detected by EMIT but at the significantly higher levels of 5.0 $mg\ L^{-1}$ and 25.0 $mg.L^{-1}$ respectively (Smith and Joseph, 1989). According to the manufacturers the assay is only designed to be used with human urine, and technical and procedural errors may interfere with the tests. Both RIA (Roche) and EMIT (EMIT Syva Corp) are available commercially as kits.

TDx FPIA is also used for the detection of BE. FPIA quantitates the amount of antibody that has been bound to a fluorescent tracer and a competing analyte by measuring the degree of rotation of plane polarized fluorescent signal emitting from a tracer molecule. According to Poklis, (1987) cocaine and EME cross reactivity with TDx assay was less than 2% and no false positive or inhibition of BE occurred. TDx is available from Abbott. (Poklis, 1987).

The advantage of general screening techniques is that they are fast and a large number of samples can be processed in a relatively short period of time which is an advantage for a busy laboratory. Although immunoassay techniques are useful for general screening, chromatographic methods are required for confirmation of identity. Isenschmid, (1988) showed that out of 35 samples, five samples which were found negative by EMIT for BE, were found positive by GC-MS. One of the samples had a concentration of $0.42 \mu g ml^{-1}$.

5.7.2. Confirmatory techniques

The confirmatory techniques include sophisticated chromatographic techniques such as GC-MS or HPLC. In some cases e.g pharmacodynamic or pharmacokinetic studies, the general screening methods may not be necessary and confirmatory techniques are used to identify unknown metabolites. e.g Zhang and Foltz (1990) reported cocaine and 11 of its metabolites in an extensive study done on a single urine sample from a cocaine user using GC with an ion-trap detector.

A large number of papers have been published using chromatographic techniques for identification and quantitation of cocaine and its metabolites. A list of analytical techniques used for various cocaine metabolites is given in TABLE 5.1.

TABLE 5.1

Metabolites	Method used
Cocaine	Colorimetry, GC-FID, TLC, GC-NPD, GC-ECD, Radioscan TLC, TDx, GC-MS, RP-HPLC,
Benzoylecgonine	Spectrophotometry, GC-FID, GC-ECD, GC-NPD, GC-MS, HPLC-GC-MS, RIA, EMIT, TDx, TLC
Ecgonine methyl ester	H^3Radioscan, GC-MS, GC-NPD, GC-FID,
Ethylcocaine	GC-MS, RP-HPLC, GC-NPD
Norcocaine	RP-HPLC, H^3 Radioscan TLC, GC-MS,
Ecgonine	TLC as butyl derivatives
Other metabolites	H^3 Radioscan TLC, GC-MS

As can be seen from the TABLE 5.1 GC-MS has been used to identify or quantify most cocaine metabolites because of its unique advantage of providing structural fingerprints. It is of interest to note that in the literature search done for this work covering the period between 1951 to 1991, the percentage use of various analytical techniques for cocaine and its metabolites was as follows:

Techniques	percentage
GC-MS	32%
GC -FID,ECD,NPD	26%
Others TLC,RIA, etc	26%
HPLC	14%

Although GC-MS has been widely used for the identification and quantification of cocaine and its metabolites, it is not the ideal technique as most of the metabolites are very polar and water soluble. The most suitable analytical technique for cocaine and its metabolites would be RP-HPLC-MS. Unfortunately due to problems with interfacing LC with MS, it is still too expensive at present to be a routine procedure.

HPLC is most suited for the detection of cocaine and its metabolites and large numbers of papers have been published including Evans and Morarity 1980, Masoud and Krupski 1980, Svensson 1986, Khan et al. 1987, Lampert and Stewart, 1989, Lau et al. 1990, Sandberg and Olsen 1990, Roberts et al. 1992, and Browne et al. 1992. Nearly all of the published methods have relied on U.V detection (which is the most commonly used detection technique) between 230-235 nm. on reversed phase columns at pH 2.0. One of the problems with U.V detection is that it is only suitable for compounds which have chromophores in their structure and thus only cocaine, benzoylecgonine, ethylcocaine, and norcocaine have been identified and quantified using this technique.

As discussed above in Section 5.5, ecgonine methyl ester (EME) is a major metabolite of cocaine and its presence and measurement with benzoylecgonine gives important information regarding the time of ingestion and more positive confirmation of cocaine use. Unfortunately it has no chromophore and its UV detection by HPLC in biological samples is very poor even at low wavelengths such as 190-200nm.

An HPLC method that measures EME has been reported by Miller and DeVane (1991) which measures cocaine and benzoylecgonine by U.V detection at 230nm and EME by electrochemical detection, with two detectors arranged in series. A mobile phase of pH 8.0 and a polymeric column was required. Unfortunately ethylcocaine was used as the internal

standard and the analytes were measured in sheep plasma in the calibration range of 100 to 2400 ng ml^{-1}.

The general screening methods rely on the presence of BE in biological samples, however it is important to note that cocaine hydrolyses slowly to BE in aqueous solutions at pH 7.4. Stewart (1979) found that 42% of a cocaine sample when incubated at pH 7.4 in buffered saline was converted to benzoylecgonine in 24 hrs. According to this author this may be a sufficient explanation for its renal excretion. EME is a more reliable indicator of cocaine use as it is produced by plasma and liver esterases, and therefore an HPLC method which can measure EME with other metabolites has a potential for being used as a more positive general screening method.

5.7.3 Sample preparation

Analysis of trace amounts of analytes of interest in samples such as biological matrices requires sample preparation to avoid the interferences which may be caused by substances which may closely resemble the analytes. Liquid-liquid extraction (LLE) and solid phase extraction (SPE) have both been used for the sample preparation of cocaine and its metabolites from biological samples such as human hair (Cone et al. 1991), blood (Hearn et al. 1991), plasma (Javaid et al. 1978), urine (Ambre et al., 1988) and meconium (Browne et al. 1992). Both the methods have their advantages and disadvantages depending upon the type of sample and the analytical technique used.

In general the liquid-liquid extraction of cocaine and metabolites from substances such as urine or blood is carried out at a basic pH such as 9.0 into a mixture of chlorinated organic solvent and an alcohol e.g chloroform and ethanol (Lau et al. 1990) or dichloromethane and isopropanol (Roberts et al. 1992). The presence of alcohol increases the recovery of hydrophilic substances (Lau et al. 1990) such as benzoylecgonine, benzoylnorecgonine and ecgonine methyl ester. The recoveries for cocaine, norcocaine and benzoylecgonine using LLE have been reported to be between 80-90% (Lau et al. 1990). However recoveries for

ecgonine methyl ester were between 40-50% (Ambre et al. 1982). Liquid-liquid extraction suffers from the problems of lack of reproducibility (Sandberg and Olsen, 1990), and contaminated blanks. Samples extracted by liquid-liquid extraction without further clean up are generally suitable for GC-MS in selected ion monitoring, this may however affect the GC column which would require regular maintainence to avoid build up of interferences and noisy baselines.

With the introduction of special cartridges by various manufacturers the use of solid phase extraction is finding increasing use by various workers. Lampert and Stewart (1989) used two cartridges, an SCX (strong cation exchange) and a C_{18} in series for sample preparation of cocaine and metabolites (BE, norcocaine, norbenzoylecgonine) from canine serum and reported recoveries exceeding 90% for most metabolites. A modified U.V detector was used to achieve a high sensitivity of about 1 ng ml^{-1}. They reported problems with interfering peaks from cartridges which were cleaned with repeated use of cleaning solvents in the preconditioning stages. Ortuno et al. (1990) using Bond Elut Certify™ and GC-NPD reported recoveries of 40% for ecgonine methyl ester with the recoveries of cocaine and BE around 90%. Sensitivity limits were 100 ng ml^{-1} for cocaine and BE and 250 ng.ml^{-1} for EME. They also found that recovery of ecgonine methyl ester from urine was increased from 20 to 40% by simply decreasing the amount of sample, which indicated that sample components were competing with the analyte for the binding sites on the cartridges.

Most cocaine metabolites, including ecgonine and ecgonine methylester are tertiary amines and can form ion-pairs with negatively charged fluorescent ion such as DAS. The post-column system which has been used in earlier chapters of this thesis offered interesting possibilities in terms of increasing sensitivity and selectivity for cocaine and its metabolites. This chapter examines the use of post-column ion-pair extraction system for cocaine and its metabolites.

5.8.Synthesis of metabolite standards

COCAINE BASE (Lampert and Stewart,1989)

Cocaine hydrochloride was dissolved in distilled water, the solution was basified with a saturated solution of sodium carbonate and extracted three times with diethyl ether. The ether layer was washed with water and dried over anhydrous magnesium sulphate. It was then filtered and evaporated to dryness. The dried residue was left overnight in a vacuum oven to dry the powder to a constant weight.

BENZOYLECGONINE (Findlay,1954)

Cocaine base (1.0 gm.) was refluxed with 100 ml of water in a round bottom flask for 24 hrs. The solution was allowed to cool and extracted twice with diethyl ether and stored overnight in a refrigerator. White needles of benzoylecgonine were collected and were dried to a constant weight in a vacuum oven over phosphorus pentoxide. About 700 mg of product was obtained whose I.R and ^1H NMR spectra resembled benzoylecgonine. The spectra are given in Appendix 1.

ETHYLCOCAINE (Ortuno et al., 1990)

Benzoylecgonine (120mg.), acetone (10.0 ml.), iodoethane (5.0ml) and potassium carbonate (1.0 gm.) were refluxed in a round bottom flask for 4.0 hrs. The mixture was evaporated to dryness, dissolved in chloroform and washed twice with water. It was again evaporated to dryness and dissolved in a small amount of methanol. A few drops of water were added until the solution became cloudy. It was then left overnight in a refrigerator. Prism-like crystals (110.0mg) of ethylcocaine were obtained. The structure was confirmed by ^1H NMR. Appendix 1. The compound was found to be 99% pure by HPLC.

ECGONINE

About 1.0gm of cocaine hydrochloride and 25 ml of 2N HCl were refluxed in a 250 ml round bottom flask for 4.0 hrs. The solution was extracted three times with diethylether and

evaporated to dryness on a rotary evaporator. The powder was left overnight in a vacuum oven to dry to a constant weight. About 350 mg of product was obtained. Its I.R spectrum agreed with the literature. Appendix 1.

ECGONINE METHYLESTER (Findlay, 1954)

Ecgonine hydrochloride (100.0 mg.) was dissolved in 10.0 ml of methanol and 0.5ml of thionyl chloride was added to it. The flask was left overnight at room temperature, then the methanol was removed using a rotary evaporator. White crystals of ecgonine methylester were obtained. The structure of ecgonine methyl ester was confirmed by ^1H NMR. TLC gave a single blue spot whose R_f value agreed with the literature (Ambre et al 1982). GC-MS showed a single peak and the mass spectrum of the compound confirmed it to be ecgonine methyl ester.

NORCOCAINE

Initial attempts were made to synthesize norcocaine following the procedure according to (Werner et al 1967). However the yield was not good. Norcocaine was finally synthesized following the procedure (Borne et al, 1977).

1) Cocaine base (3.18 gm.) was dissolved in 25 ml. of toluene.

2) 2.41 gm. of 2,2,2, trichloroethylchloroformate was dissolved in 25.0 ml of toluene.

Both the solutions were combined and the resulting solution was refluxed for 18 hrs. The mixture was cooled to 5° C using ice and 0.2 ml of formic acid was added to it. After stirring for another 30 min, 0.4 ml of triethylamine was added and the resulting mixture was stirred at room temperature for 1.0 hr. Water (25.0 ml.) was added and the mixture was extracted with ether (3 X 50 ml) .

The ether extracts were combined and extracted with 175 ml of 5N HCl to remove any unreacted cocaine. The ether layer was dried over anhydrous magnesium sulphate and evaporated to dryness to yield a viscous oil. The oil was dissolved in 12.5 ml of dimethylformamide (12.5 ml) and cooled to 5° C. Formic acid (0.8 ml.) was added, followed

by addition of zinc dust (1.59gm) in portions while maintaining the temperature to 5°C. After addition of zinc dust the mixture was stirred for another 18 hrs. The mixture was filtered, and the filtrate was poured onto 100 gm of crushed ice. When cold, the mixture was made strongly acidic with concentrated hydrochloric acid and extracted with ether (2 X 50 ml). The acidic layer was again cooled to 5° C and made strongly alkaline with ammonium hydroxide and extracted with ether (2 X 50 ml). The ether extract was combined and dried over anhydrous magnesium sulphate and evaporated to give a viscous oil, which was then titurated with petroleum ether to give a solid. The solid was then dissolved in ethereal HCL to give 1.0 gm of norcocaine hydrochloride. Mpt. 112°. The structure was confirmed by ^{1}H NMR and mass spectroscopy. Appendix 1.

5.9. Results and discussions

The approach for the development of a sensitive HPLC method for cocaine and its metabolites was to use post-column ion-pair formation with fluorescence detection, so with this in mind the chromatography of cocaine and its metabolites was investigated. Most of the published procedures were not suitable for use with post-column ion-pair extraction systems as they had used silanol masking agents such as TEA (triethylamine) or TBAH (tetrabutyl ammonium hydroxide) to suppress the tailing of basic compounds on reversed phase columns.

The published procedure that was adopted as a starting point was that of Jatlow et al., (1978) which used a mobile phase of ACN - 0.25 M phosphate buffer, pH 2.8 on an ODS column. The ODS column was replaced by a phenyl column because it is less hydrophobic and requires lower amounts of organic modifier to give small κ' values and therefore give sharper and narrower peaks which are suitable for post column work.

Keeping the ACN concentration constant at 15% and using phosphate buffer the chromatography of cocaine and benzoylecgonine on a phenyl column was investigated at 220 nm. The results are shown in TABLE 5.2.

TABLE 5.2

Ionic strength	pH	cocaine (κ)	BE (κ)
0.25 M	2.8	2.90	1.45
0.1 M	4.0	3.18	1.36
0.05M	4.0	3.36	1.36

Under these conditions it was quite possible to detect 0.16 µg/ml of cocaine and benzoylecgonine using a 10µl loop. Ecgonine methyl- ester eluted with the solvent front and was poorly detectable (100µg/ml) at 200 nm.

5.9.1. Preliminary studies for post-column system

Cocaine is highly lipophilic and is readily soluble in chlorinated organic solvents. Benzoylecgonine is amphoteric and its extraction into organic solvents is a major problem. To see how these compounds would behave under post-column conditions a flow injection system as described in Chapter 3 was used i.e. a post-column system without the column. TABLE 5.3 shows the result of these studies and indicate that under the conditions used, the benzoylecgonine ion-pairs were poorly extracted by chlorinated organic solvents. The addition of lipophilic alcohols to the chlorinated organic solvent improved the extraction of BE -ion-pairs thereby increasing the sensitivity. TABLE 5.4 shows the effect of ionic concentration on the extraction of cocaine and benzoylecgonine

The results show that under the experimental conditions the ionic concentration of buffer did not influence the extraction of cocaine and benzoylecgonine when varied from 0.1-0.05M.

TABLE 5.3
Aqueous phase: 0.25M phosphate buffer pH 4.0
Organic phase : varied
Reagent (DAS) concentration : varied

Organic phase	[M] of reagent	Cocaine Pk ht in mm n=5	BE Pk ht in mm n=5
DCE	$5.84 X10^{-5}$	110.2 (4µg/ml)	n.detected
DCE	$1.40 X 10^{-4}$	163	6 (20µg/ml)
DCE + 5% pentanol	as above	over range	60 (20µg/ml)
Chloroform	as above	--	5 (0.38mg/ml)
DCE +10% pentanol	as above	--	noise too great
as above	$5.84 X 10^{-5}$	--	60 (20µg/ml)

TABLE 5.4
Aqueous phase : As in the table
DAS concentration : 5.87 X 10^{-5} M
Organic phase: DCE +5%pentanol

Aq.phase	cocaine Pk.ht. (mm) n=6	BE Pk ht. (mm)
0.25M pH 4.0	146	30
0.1M pH 4.0	156	45
0.05M pH 4.0	156	45

Schill (1974) has shown that the addition of a lipophilic alcohol e.g pentanol improves the extraction of hydrophilic substances into chlorinated organic solvents. Nielsen (1974) using batch extraction studies showed that the extraction of hydrophilic substances i.e carboxylic acids as ion-pairs was improved by the addition of Crown ethers (18-Crown-6) into chloroform. The Crown ethers are cyclic polyethers with electron donating properties and form complexes with carboxylic acids in organic solvents such as chloroform, and this complex formation improves the extraction of acids and other hydrophilic compounds from aqueous solutions (Nielsen and Modin, 1973) to chlorinated organic solvents. TABLE 5.5 shows the results when the organic phase was varied while keeping the reagent concentration and aqueous phase constant

TABLE 5.5
Aq Phase 0.05 M pH 4.0
DAS 5.87 X 10^{-5} M
Organic phase varied

organic phase	BE Pk ht (mm)	Ecgonine Pk ht (mm)	EME Pk ht (mm)
DCE +10% pentanol	42 ± 3 mm MDL 10µg ml^{-1}	70 ± 3 mm 55µg/ml	133 ± 2 mm 58 µg/ml
DCE + 12% pentanol	Background too high		
DCE +1X10^{-2} M $D_{18}C$	Background too high		
DCE + 1X10^{-4} M $D_{18}C_6$	Background too high		
as above with DAS reduced to 2X10^{-5}M	Background high 206 µg/ml		

The results showed that although DCE with 10% pentanol gave better peak heights than with 5 % pentanol it also gave a higher background noise. Crown ethers also gave higher background. and were therefore not suitable in this case.

Changing the length of extraction coil from 1.0 metre to 2.5 metre did not make any significant difference.

Considering the nature of the metabolites and the results from the above experiments it was realised that the experimental conditions selected were not suitable for the enhanced detection of benzoylecgonine, ecgonine and ecgonine methylester, and the experimental conditions were therefore modified.

It was realised that the poor extraction of benzoylecgonine, ecgonine and ecgonine methylester as ion-pairs into chlorinated organic solvents in a post-column ion-pair extraction system was caused by the presence of hydrophilic groups such as the -COOH of BE and ecgonine the -OH group in the case of ecgonine methylester. Therefore in order to improve the chromatography and to enhance the fluorescence detection of these compounds precolumn derivatization was investigated.

5.9.2 Precolumn derivatization

The metabolites of cocaine i.e BE, ecgonine, ecgonine methylester are very polar and are quite difficult to retain under reversed phase conditions with predominantly aqueous mobile phases. This problem becomes more complicated when these metabolites are present in polar matrices such as biological samples because no matter how effective the sample clean up is, some sort of interferences will show up in the chromatogram. Derivatization of analytes is one way of getting a comparatively clean chromatogram as this increases the hydrophobicity of the polar analytes, increasing their retention time and pulls them away from the early eluting peaks of the sample matrix.

The -COOH groups of BE and ecgonine can be easily derivatized, likewise the -OH groups of EME and ecgonine can also be derivatized with suitable reagents to form esters. The esters being hydrophobic compared to the parent compound would be easily retained on the column and enhance detectability.

As a post-column ion-pair extraction system was being used for the detection of analytes by fluorescence, pre-column derivatization reagents that introduce non-fluorescent groups were preferred over the labelling ones. According to Fruijiter et al (1990) the GC derivatization techniques e.g alkylation, acylation or silylation may be used in HPLC using the same or different reagents.

A. Derivatization with DMF

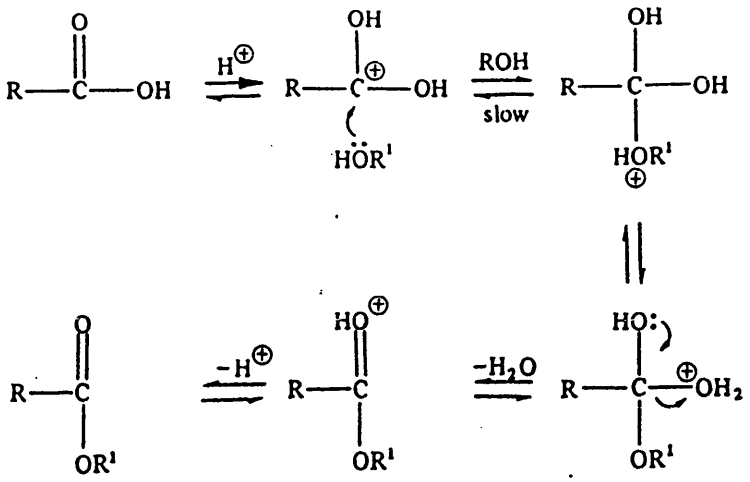
Initial attempts were made to derivatise BE with DMF-DMA (dimethyl formamide dimethyl amine) which has been used successfully to derivatise compounds containing a -COOH group to the methyl ester for gas chromatographic analysis. But it was soon found that the reagent was not suitable for HPLC because the methyl ester was hydrolysed as soon as aqueous mobile phase was added to it (Hulshoff and Forch, 1981).

$$RCOOH + (CH_3)_2N\text{-}CH\,(OR')_2 \longrightarrow RCOOR' + R'COH + (CH_3)_2N\text{-}CHO$$

B. Alkylation reactions

These involve the replacement of an active hydrogen group by an alkyl group. Compounds with primary, secondary or tertiary hydroxy groups, phenols, and carboxyl groups among others can be derivatised by this method resulting in the formation of less polar compounds . The alkylation reactions may be acid catalysed or base catalysed.

Acid catalysed alkylation reaction require the use of acidified alcohols i.e low molecular weight alcohols containing H_2SO_4 or alcohol through which HCl gas has been bubbled. Sometimes instead of HCl gas acetyl or thionyl chloride is used. The general mechanism has been summarised by Hulshoff and Lingeman (1984) as:



Acid enhances the carbonyl character of the RCOOH by protonation, thus rendering the carbonyl atom more susceptible to nucleophilic attack. It also has the effect of promoting the loss of water as H_2O is more easily lost than hydroxyl ions. Methylation is the first choice but other esters can be prepared.

Methylation of benzoylecgonine was ruled out as cocaine is methyl benzoylecgonine and so more hydrophobic esters were considered.

a Derivatization with acidified ethanol

Attempts were made to prepare the ethyl ester derivative of benzoylecgonine using ethanol and H_2SO_4. The chromatograms contained many extra peaks apart from the ethyl ester peak and also had the disadvantage of making the sample too acidic, making the adjustment of pH difficult before injection.

Ethanolic HCl (dry HCl gas bubbled through absolute ethanol) proved to be a suitable reagent for derivatisation of benzoylecgonine. The chromatograms were cleaner and the pH of the sample could be easily adjusted to that of the mobile phase.

Two derivatization studies were carried out, monitored by HPLC

1) Effect of heating time on derivatisation of benzoylecgonine.

2) Effect of reagent volume.

The chromatography was carried out on a Phase Sep Cyano column (100 X 4.6 mm i.d)

Conditions :

Mobile Phase A: Acetonitrile - 0.05M phosphate buffer pH 4.0 (10:90v/v)

Mobile Phase B: Acetonitrile 100%

A: B 90:10 at 1.0 ml min^{-1}

Column : 100 X 4.6 mm i.d 3µ Phase Sep Cyano.

Detector : U.V at 205 nm.

1.Effect of heating time on derivatization of benzoylecgonine

Sample : 1.0 ml of ethanolic solution of BE (15 µgml^{-1}) with 1.0 ml of ethanolic HCl .

Control : 1.0 ml of ethanol + 1.0ml of ethanolic HCl

Sample and control were prepared and heated in a hot air oven at 110° C for varying periods of time, evaporated to dryness, reconstituted in 1.0 ml of mobile phase, vortexed for 30 seconds and injected. The results are shown below in TABLE 5.6.

TABLE 5.6

Time (min)	EBE Pk ht (mm)	BE Pk ht (mm)
0	0	158
15	6	139
30	14	124
45	21	103
60	33	70
120	42	12
180	43	--
240	35	--

2. Effect of reagent volume

Chromatographic conditions as before

Samples were prepared by adding varying volumes of ethanolic HCl to 1.0 ml. ethanolic solutions of benzoylecgonine (15μg ml^{-1}) in a (5ml) Reacti-vial. After heating at 110^0 C for 2.0 hrs, the samples were allowed to cool, evaporated to dryness on a water bath under a gentle flow of N_2, mobile phase added, vortexed for 30 seconds and injected. The results are given below:

TABLE 5.7.

Volume of Ethanolic HCl (ml)	BE Pk. ht. (mm)	EBE Pk. ht (mm)
0.5	20	31
1.0	n.d	39
2.0	n.d	32[a]
3.0	n.d	25[a]

[a]A large no of extra peaks and charring, n.d not detected.

The results from both these studies showed that the best conditions for derivatization of BE to its ethylester were 1.0 ml of ethanolic HCl and heating for 1.5 hrs. Heating for 2.0 hrs produced extra peaks and charring, resulting in lower yields. It was easily possible to detect 0.15 μg/ml of benzoylecgonine (ethylester) under post-column conditions using dichloroethane as the extraction solvent. Benzoylecgonine eluted after cocaine.

Attempts were then made to derivatise the COOH group of ecgonine. As ecgonine is more polar it eluted with the solvent front thus making it very difficult to quantify the derivatisation procedure. It was reasoned that if the COOH group could be butylated it would be easier to quantify the derivatisation procedure. Another advantage of making the butyl ester was that it is not a metabolite of cocaine whereas ethyl ester of benzoylecgonine (ethylcocaine) exists as a metabolite in persons who concurrently abuse ethanol and cocaine.

b. Derivatization with butanolic HCl

To quantify the derivatisation procedure for ecgonine a C_8 (SGE 100 X 2.0 mm i.d.) column was used as it is more hydrophobic than the cyano column.

Derivatisation procedure:

Ecgonine (2.0 mg) was dissolved in 1.0 ml of methanol,100 µl of it was diluted to 10.0 ml (20 $\mu g ml^{-1}$).

1. 1.0 ml of methanolic solution of ecgonine (20.0 $\mu g\ ml^{-1}$) was evaporated to dryness in a Reacti-vial 1.0 ml to dryness under N_2.

2. 1.0 ml of butanolic HCl (butanol through which dry HCl gas has been bubbled until saturation) was added to the dried residue.

3. The vials were heated in an oven at 100°C for various time intervals as shown below.

4. The vials were allowed to cool and evaporated to dryness under N_2.

5. The sample was dissolved in the mobile phase.

For each sample a control was carried out which used plain methanol evaporated to dryness. The effect of heating time on the formation of butylecgonine was measured at 0.5-2.0 hrs. at 200 nm (U.V) and under post-column conditions. The results are shown in TABLE 5.8

TABLE 5.8

Column C_8 (100 X 2.0 mm i.d)

Mobile Phase: Acetonitrile - 0.05 M phosphate buffer pH 4.0 (20:80 v/v)

Flow rate = 0.2 ml min^{-1} , Temp 30°C.

Detection at 200 nm[a]				Post-column Fluorescence detection [b]		
Time	Ret. time (min)	Pk. ht (mm)	$w^{1/2}$ (mm)	Ret. time (min)	Pk. ht (mm)	$w^{1/2}$ (mm)
0.5	12.4	44	3.2	12.6	27	3.2
1.0	12.4	57	3.0	12.6	36	3.6
2.0	12.4	97.5	2.8	12.4	61	3.0

[a] 0.02 aufs b at Range 100

The results showed that 2.0 hrs heating at 100°c was sufficient for derivatisation of ecgonine to butyl ecgonine. It can also be seen from the table that there is very little difference in band width at half height between U.V detection at 200 nm and under post - column conditions.

C. Chromatography on C_8 column

A simple isocratic system was not suitable for chromatography of the butyl esters of benzoylecgonine and ecgonine. The butyl ester of benzoylecgonine was more hydrophobic and gave longer retention times compared to the butyl ester of ecgonine. In order to reduce the retention of butyl ester of benzoylecgonine a higher percentage of organic modifier was required. A step gradient was therefore used for the chromatography of both the metabolites. The calibration curve of the standards was prepared in the mobile phase using methadone as an internal standard.

Mobile Phase

A : Acetonitrile-0.05M Phosphate Buffer pH 4.0 (10:90 v/v)

B: Acetonitrile-0.05M Phosphate Buffer pH 4.0 (50:50 v/v)

Organic solvent: Dichloroethane

DAS Conc: $1X10^{-4}M$

Gradient conditions

Time (min)	% A	% B	Flow rate ml min^{-1}
1.0	80	20	0.2
6.0	13	87	0.2
18.0	13	87	0.2
18.1	80	20	0.2
23.0	80	20	0.2

Ecgonine (as butyl ester)

Conc ($\mu g ml^{-1}$	Pk.ht (mm)	I.S Pk ht (mm)	Ratio
1.35	14	75	0.186
2.70	34	75	0.453
5.40	67	67	1.0
10.80	78	40	1.95
13.50	94	32	2.61

Benzoylecgonine (as butyl ester)

Conc ($\mu g\ ml^{-1}$)	Pk. ht. (mm)	Pk. ht. ratio
1.1	21	0.28
2.2	55	0.73
4.4	105	1.57
8.8	127	3.18
11.0	140	4.38

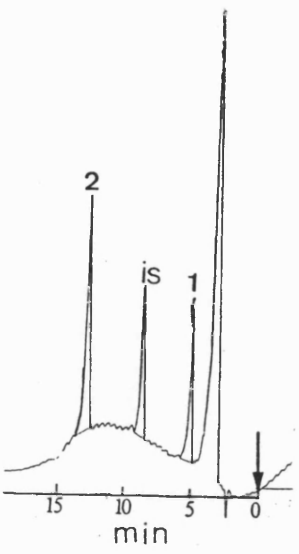


Fig 5.3 (1) Ecgonine 2.70 μg ml^{-1}, and (2) benzoylecgonine 2.2 μg ml^{-1}, as butylesters, conditions as specified in the text, (is) methadone.

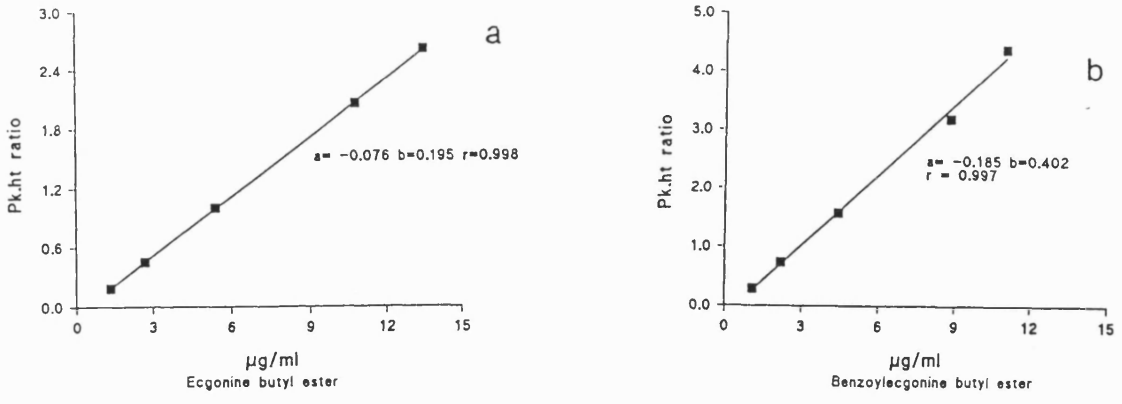


Fig 5.4 Calibration plots of (a) Ecgonine and (b) BE as butylesters, conditions as Fig 5.3.

For benzoylecgonine and ecgonine the calibration curves were linear in the range tested. For BE a= - 0.185, b = 0.402, r =0.997 , ecgonine a = - 0.076, b=0.195, r = 0.998. The intercepts were not significantly different from 0 at n-2 df. at 95 % confidence limit. Fig 5.3 shows a sample chromatogram and Fig. 5.4 shows the calibration plots of ecgonine and benzoylecgonine as their butylesters.

Chromatography of cocaine and norcocaine:

The chromatographic conditions that were suitable for the separation of butyl esters of benzoylecgonine and ecgonine were not suitable for the chromatography of cocaine and norcocaine as they both eluted together. Changing the gradient conditions, mobile phase pH, or ionic strength did not improve the chromatography. Changing the organic phase from acetonitrile to methanol increased the retention and both compounds eluted as badly tailing peaks.

When an isocratic system was used with acetonitrile as the organic modifier cocaine and norcocaine were separated. The results are shown in TABLE 5.10

TABLE 5.10

Mobile phase : 0.05 M phosphate Buffer pH 4.0

Percentage of ACN	k' Cocaine	k' Norcocaine
20	8.5	10.5
30	6.2	6.5
40	3.6	3.6

As it was possible to separate cocaine and norcocaine under isocratic conditions but not under gradient conditions, the reason was considered to be due to the large delay volume of the gradient system (5 ml delay volume at 0.2ml min^{-1}). This made it impossible to select a successful gradient. To reduce the delay time the gradient controller was modified at a later stage as discussed in Chapter 2. of this thesis.

From these results it was concluded that with the present system in order to separate all the metabolites of cocaine a different retention mechanism was needed.

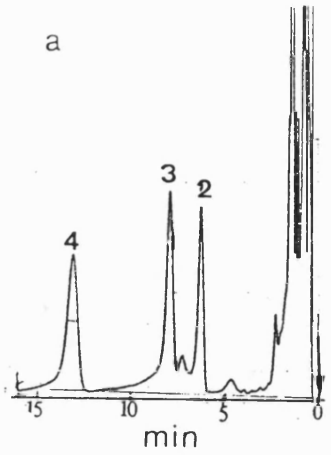
D. Chromatography on ion-exchange columns

Ion exchange columns are packed with materials which contain covalently bonded ionic functional groups. These are either acidic groups such as sulphonic acid (SCX) or carboxylic acids (WCX) i.e strong or weak cation-exchange resins for separation of cations and amines, or are quaternary ammonium (SAX), or amino groups (WAX) i.e, strong or weak anion exchange groups for separation of anions and acids. The separation of samples is based on the strength of interaction between the sample ions and the ion- exchange sites. Ions that interact weakly with the ion exchange site are poorly retained and have small κ' values while the ions that have a strong affinity for ion exchange sites have high κ' values. The retention on an ion-exchange column is controlled by varying the ion concentration of the eluent either by varying the ionic concentration of the buffer or by adding a neutral salt.

As described above in Section C cocaine and norcocaine were not separated on a C_8 column nor on a C_{18}. A 50 X 4.6 mm i.d column packed with Nucleosil SA (a strong cation exchange) was tested. Fig 5.5 (a) shows a chromatogram of cocaine, norcocaine, and the butylester of benzoylecgonine on this column at 205 nm.

However it was not possible to use this column in combination with the post-column ion-pair extraction detector because of the high organic content used in the mobile phase (i.e., over 50%) resulting in a high background signal. A 2.0 mm i.d column was therefore ordered from the manufacturers packed with the same packing material. Problems were experienced in getting an efficient ion exchange 2.0 mm i.d narrow bore column. Fig 5.5 (b) illustrates a chromatogram obtained on the best 2.0 mm i.d Nucleosil ion-exchange column at 205 nm. As can be seen from the chromatogram 2.0 mm i.d

Fig 5.5 (a) Chromatogram on Nucleosil SA 50 X 4.6 mm i.d column at 205 nm, with ACN-0.05 M phosphate buffer pH 4.0 (50:50 v/v) (2) norcocaine, (3) cocaine (4) BE as butyl ester.



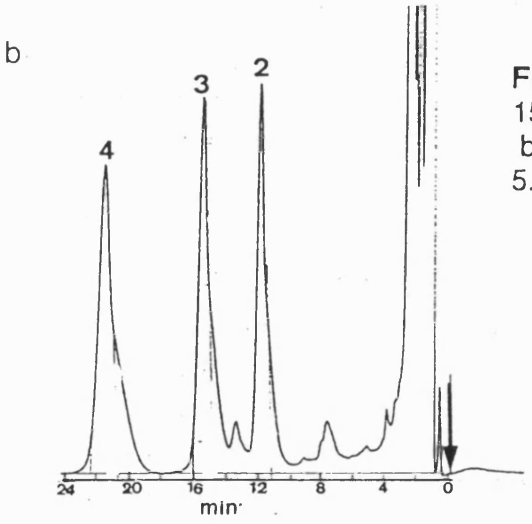
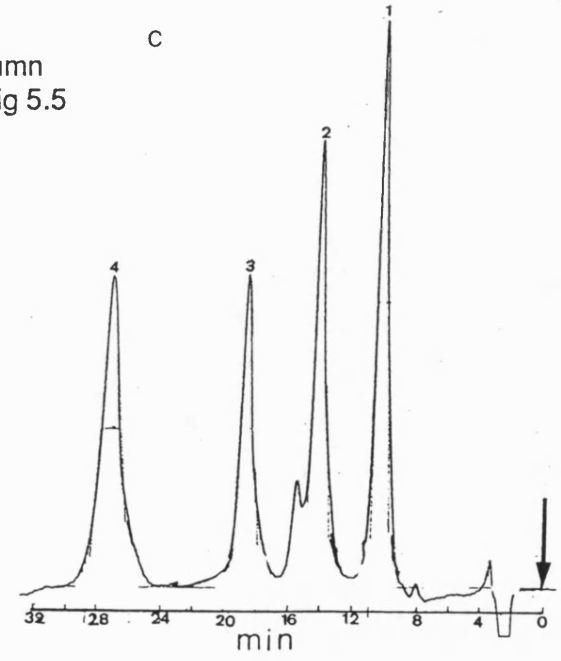


Fig 5.5 (b) Chromatogram on Nucleosil SA 150 X 2.1 mm i.d, with ACN-0.1M phosphate buffer pH 4.0 (30:70 v/v), codes same as Fig 5.5 (a)

Fig 5.5 (c) Chromatogram, with post-column ion-pair detection, conditions same as Fig 5.5 (b) codes (1) ecgonine butylester.



column is giving a reduced plate height of 8.82, which is highly undesirable. Fig 5.5 (c) is a chromatogram of cocaine metabolites under post-column conditions. Note that ecgonine is easily detected under these post-column conditions.

Sample preparation

Using the procedure published by Varian Associates (See Chapter 2), the Bond Elut Certify gave good recoveries for cocaine, norcocaine and BE of 90, 92. and 77 % respectively. However ecgonine was not retained on the cartridge because of its extremely polar character. Stewart and Lampert (1989) had used an SCX cartridge for the sample preparation of cocaine metabolites. It was thought that it may be possible to retain ecgonine on an SCX cartridge. However, no conclusive results could be drawn from this approach as the eluting buffer i.e 50:50 methanol-0.5M sodium carbonate buffer pH 10.0 was not compatible with the acidic derivatization conditions. Using an eluting buffer with increased ionic strength at a higher pH did not make much improvement. As ecgonine is very minor metabolite in urine (about 3%) it was dropped from the study.

E. Derivatization of ecgonine methylester.

The derivatization of ecgonine methyl ester was necessary in order to improve its chromatographic properties as it is very polar and eluted with the solvent front. Ecgonine methyl ester contains a hydroxy group which can be easily derivatized with a suitable derivatizing reagent.

Silylation is widely used in GC for derivatization of compounds which contain hydroxy groups to form trialkylsilyl ethers. Most of the silylating agents which are used in gas chromatography are very sensitive to moisture and the derivatives are also susceptible to hydrolysis and therefore cannot be used in RP-HPLC. Tertiary butyldimethyl siloxy groups (TBDMS) are 10^4 times more stable to hydrolysis than tertiary methylsiloxy groups. TBDMS functional groups have been used for the protection of hydroxy groups during the synthesis

of many compounds including ribonucleosides, deoxyribonucleosides, carbohydrates, Corey and Venkateswarlu (1972), Mawhinney and Madson (1982).

Initial attempts to derivatize EME were made following the procedure of Corey (1972) where 1.2 meq of dimethyl tert-butyl silyl chloride and imidazole (2.5 meq) were added to an acetonitrile solution of EME and left at room temperature for 48 hrs. The acetonitrile was evaporated to dryness and TLC showed a single spot at 25 mm (EME at 10 mm) following Ambre et al. (1982). The structure of the derivative was confirmed by GC-MS, as shown in Fig 5.6. Cocaine and norcocaine were not silylated by this procedure. As the initial experiment was successful, MTBSTFA reagent was used for the derivatization of EME. The advantage of choosing MTBSTFA was that the reaction takes place in a few minutes (Mawhinney and Madson 1982) and imidazole is not required as a catalyst which may interfere with post-column ion-pair extraction detection. Fig 5.7 shows a chromatogram of cocaine on an ion-exchange column spiked with EME (derivatized with MTBSTFA).

F.Derivatization of Benzoylecgonine and Norcocaine with alkyl halides

It was realized that the BE and EME could not be derivatized in a single step. Two steps were necessary:

Step 1 : Derivatization of the COOH group of BE with butanolic HCl

Step 2: Derivatization of EME with MTBSTFA.

Step 1 utilized butanolic HCl and it was realized that the presence of even a small amount of butanol would interfere with step 2 i.e., derivatization of the OH group of EME with MTBSTFA. The complete removal of butanol was found to be a lengthy process. Another problem with the use of butanol was that cocaine could be transesterified to butyl-benzoylecgonine. To overcome these problems another derivatization procedure was required for derivatization of COOH groups.

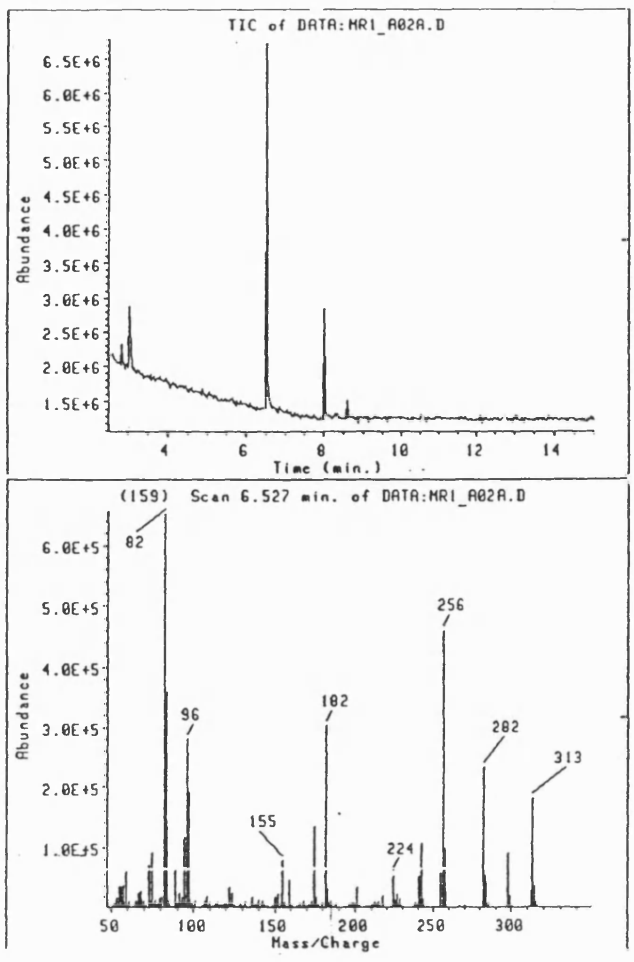


Fig 5.6 GC-MS of EME derivatized with MTBSTFA.

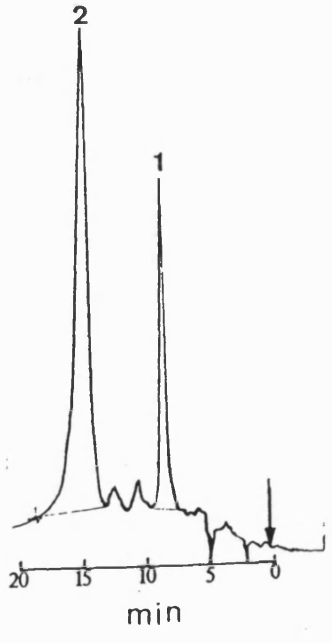
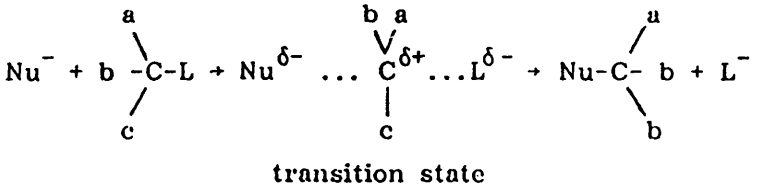


Fig 5.7 EME derivatized with MTBSTFA (2), spiked with cocaine (1) on an ion-exchange column with post-column detection.

Derivatization with alkyl halides

Derivatization of COOH acids with alkyl halides is a base-catalyzed alkylation reaction and does not require the use of low molecular weight alcohols. An iodoalkane is reacted with an acid in the presence of a base. The general mechanism of this reaction is:



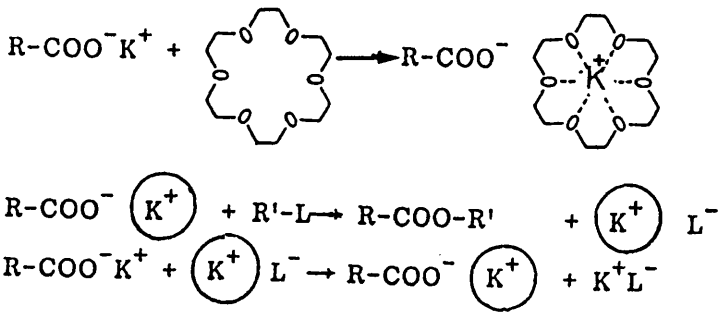
Nu = nucleophile L= leaving group

For the alkylation with alkylhalides, primary alkylhalides are used and the reaction is S_N2 type. Alkyliodides are preferred over chlorides or fluorides. For alkylation reactions, bases are used as catalysts and these can be inorganic bases like K_2CO_3 or organic bases like tertiary amines or quaternary ammonium ions as they do not undergo the alkylation reaction. Inorganic bases like K_2CO_3 form a potassium salt of the carboxylate ion which is easier to remove as shown: (Fruijiter et al 1990).

$$2RCOOH + K_2CO_3 \longrightarrow 2RCOO^- K^+ + H_2O + CO_2$$

$$RCOO^- K^+ + R_1 L \longrightarrow R\text{-}COO\text{-}R_1 + K^+\text{-} L$$

Crown ethers are sometimes used as catalyst along with K_2CO_3 to speed up the reaction as the naked ion can undergo faster alkylation as shown :



The alkylation reaction with alkylhalides is carried out in aprotic solvents such as acetone, acetonitrile and dimethylsulfoxide, because in aprotic solvents there is no possibility of hydrogen bonding and nucleophilic substitution is easier.

A form of alkylation reaction is extractive alkylation or phase transfer catalysis, the acidic compound with an excess of alkylhalide is extracted as an ion-pair with tetra-alkylammonium ions from an aqueous solution at a suitable pH into an aprotic solvent like DCM (Nicholson,1978)

A base catalysed alkylation reaction was considered suitable for the derivatization of benzoylecgonine because 1) no OH groups were involved which may interfere with the second derivatization procedure i.e derivatization of hydroxy group of EME. 2) acetone was easier to remove than butanolic HCl. Although extractive alkylation offered interesting possibilities it was felt that the quaternary ammonium compound would interfere with the post-column ion-pair extraction step and its removal prior to injection of samples would be a problem.

Ortuno et al (1990) used a base catalysed alkylation reaction in combination with a silylation reaction for the measurement of BE and EME using a GC-NPD. In their work they used ethyl iodide for making BE ester in the presence of K_2CO_3 using acetone as the solvent. Acetone was easier to remove and the reaction conditions were also compatible with the second derivatization step. With these considerations in mind, derivatization of BE was carried out using butyl iodide instead of ethyl iodide. A further advantage of alkyl halide derivatization was that primary and secondary amines were also derivatized using this procedure meaning that norcocaine would also be derivatized i.e., become more hydrophobic and its retention would increase.

The following procedure was initially adopted for the derivatization of cocaine metabolites.

METHOD A: (Ortuno et al, 1990)

1. A stock solution of all the cocaine metabolites (1 ml) was evaporated to dryness in a Reacti-vial using nitrogen.

2. To the dried residue 10 mg of anhydrous K_2CO_3 was added followed by 180 µl of acetone and 30 µl of iodobutane and the mixture vortexed for 30 seconds.

3. The vial was heated in an oven at 60° C for 3 hrs.

4. The acetone was transferred to another vial leaving the K_2CO_3 behind. The K_2CO_3 was washed with 3 X 200 µl portions of acetone, and the washings added to the orignal solution.

5. The acetone extract was evaporated to dryness under a gentle stream of N_2.

6. To the dried residue was added 100 µl of acetonitrile and 50 µl of MTBSTFA and the mixture vortexed for 30 seconds.

7. The solution was left overnight or left in an oven at 60°C for 1 hr.

8. The acetonitrile was evaporated to dryness under a gentle stream of N_2.

9. To the dried residue, 1.0 ml of mobile phase was added and the sample injected on to the HPLC.

Fig 5.8 shows the chromatogram of all the cocaine metabolites following the above procedure on a Spherisorb SCX column. MTBSTFA did not interfere either with cocaine or norcocaine. The ion-exchange column was not very efficient as can be seen from the chromatograms and a much better column was therefore needed.

G. Chromatography on base deactivated column

As discussed in the introduction (Chapter 1) base deactivated columns are marketed for the chromatography of basic compounds without the use of amine modifiers. Suplex pKb 100 is one of many base deactivated columns. A 250 X 2.1 mm i.d column was obtained from the manufacturer. Fig 5.9 shows a chromatogram of all the cocaine metabolites on this column using a mobile phase consisting of 25% acetonitrile in 0.1M KH_2PO_4 adjusted to pH 4.0 with phosphoric acid. Almost all the compounds eluted as symmetrical peaks and the column had a very good efficiency N=11218 for a 0.25 m column or 44874 plates/meter. Norcocaine after derivatization elutes after ethylcocaine but without derivatization elutes before cocaine.

As cocaine eluted too near the solvent front, the percentage strength of acetonitrile was reduced in the mobile phase. TABLE 5.11 shows the results of the study.

TABLE 5.11

	κ'					
% ACN	ionic strength	Cocaine	EC	NC	BBE	EME
25	0.1	0.27	0.60	1.33	2.43	4.36
23	0.05	0.53	0.86	1.80	3.20	5.40
21	0.05	0.60	1.10	2.00	5.10	9.0

EC =ethylcocaine, NC = butyl-norcocaine, BBE = butyl-benzoylecgonine, EME = TBDMS derivative of ecgonine methyl ester

As expected lowering the percentage of acetonitrile in the mobile phase increased the retention of more hydrophobic analytes like TBDMS-derivative of EME than cocaine.

A gradient system was therefore necessary to separate the early eluting peaks like cocaine from sample components which may interfere with the analysis and at the same time bring forward the late eluting derivatized BE and EME peaks to a manageable analysis time. With post-column ion-pair extraction systems gradient elution has to be used with caution as increasing the percentage of organic modifier in the mobile phase during a run increases the solubility of the fluorescent ion-pairing agent, i.e DAS in this case in the chlorinated organic extracting solvent, leading to an increase in the background signal giving a characteristic hump in the base line.

A modified gradient system as described in Chapter 2 of this thesis was used. A step gradient was considered most practical given the chromatographic behaviour of the analytes and the limitations imposed by the instrument.

The gradient conditions used were as follows:-

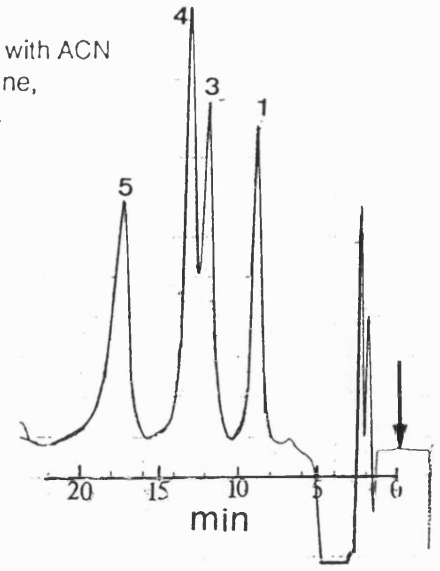
A : 15% Acetonitrile-0.05M KH_2PO_4 (v/ v) adjusted to pH 4.0

B: 28% Acetonitrile-0.05M KH_2PO_4 (v/ v) adjusted to pH 4.0

Time	%A	%B	Flow rate
1.0	100	0	0.4
4.0	100	0	0.4
5.0	0	100	0.4
17.0	0	100	0.4
17.01	100	0	0.4
22.0	100	0	0.4

Fig 5.10 shows a chromatogram of cocaine and its metabolites using the above gradient. N for a 0.25m column calculated on EME peak was 14198.

Fig 5.8 Chromatogram on Spherisorb SCX column with ACN 0.1 M phosphate buffer pH 4.0 (30:70 v/v),(1) cocaine, (3) norcocaine (4) butyl-BE (5) EME-silyl derivative.



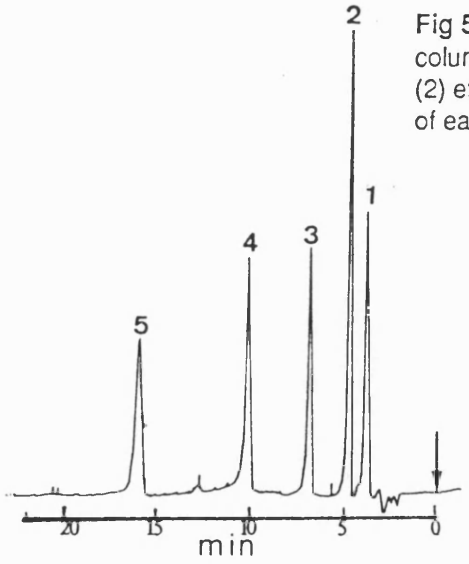
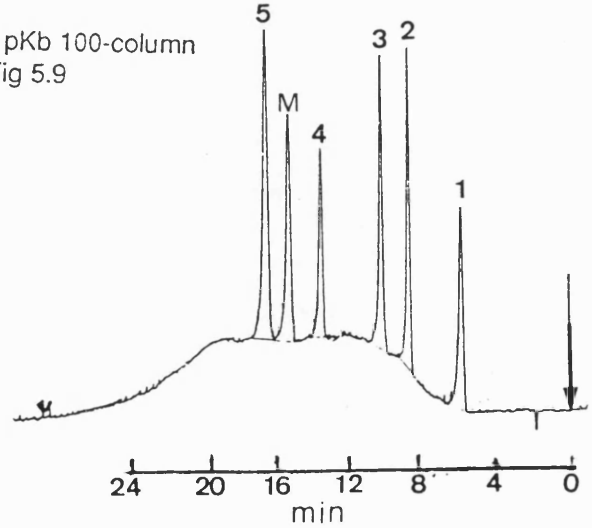


Fig 5.9 Cocaine and metabolites on Suplex pKb-100 column, with ACN-0.1M phosphate buffer pH 4.0 (25:75 v/v) (2) ethylcocaine, other codes same as Fig 5.8; 20-50 ng of each analyte on column (20µl loop).

Fig 5.10 Gradient elution Suplex pKb 100-column M=methadone, codes same as Fig 5.9



5.9.3 Optimisation of derivatization procedure

A. Alkylhalide derivatization of benzoylecgonine and norcocaine

The work done so far had been carried out using the conditions of Ortuno et al (1990) as described earlier. A three hour derivatization procedure was considered to be a major disadvantage as it decreased the sample through-put. Increasing the temperature from 60° C to 80° C using a hot air oven was not possible practically as the vial caps were not very secure at this temperature. For practical purposes 60°C was the best.

The derivatization time of 3 hrs for alkylation meant that from sample preparation to manual injection of 6 samples using gradient elution would require around 3 days for analysis. Any reduction in sample preparation time was therefore considered important to reduce the analysis time. Ideally automated sample injection would permit overnight runs and increase productivity considerably.

Crown ether i.e 18-Crown-6 has been used as a catalyst in the alkylation of fatty acids as phenacyl esters using α,p-dibromoacetophenone (Durst et al 1975). 18-Crown-6 was selected to speed up the reaction. Acetonitrile was used as a solvent so that the reaction temperature could be raised to 85°C and the vial caps remain secure. Another advantage of using acetonitrile was that it was more compatible with the silylation step. Crown ethers have one disadvantage in that they are soluble in dichloromethane and produce a large peak near the solvent front as shown in Fig 5.11 so that a minimum amount of crown ether was necessary. Reducing the amount of crown ether from 2.0 mg ml^{-1} to 4.0μg ml^{-1} did not significantly effect the derivatization yield. At 2.0 μg ml^{-1} the derivatization yields for BE and norcocaine were reduced to 73%. The use of crown ether reduced the derivatization time from 3 hrs to 1 hr. Fig 5.12 shows a sample chromatogram of alkylhalide derivatization using crown ether

Increasing the amount of iodobutane or K_2CO_3 as described earlier in Method A did not have any influence on derivatization yield or time.

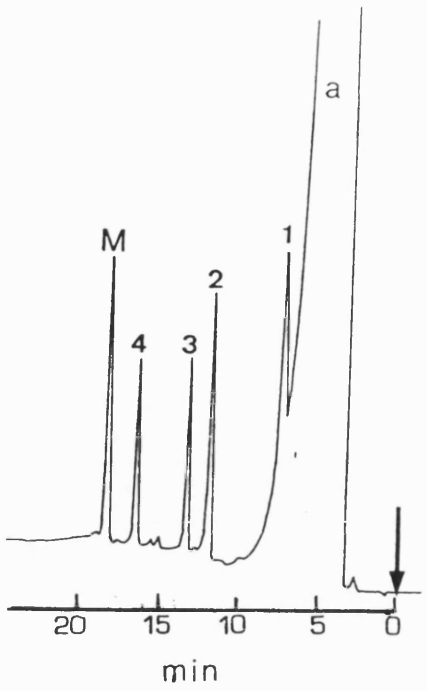


Fig 5.11 Derivatization of cocaine metabolites using crown ether as a catalyst (a) crown ether, (2) ethylcocaine, (3) butyl norcocaine, (4) butyl BE , (M) methadone.

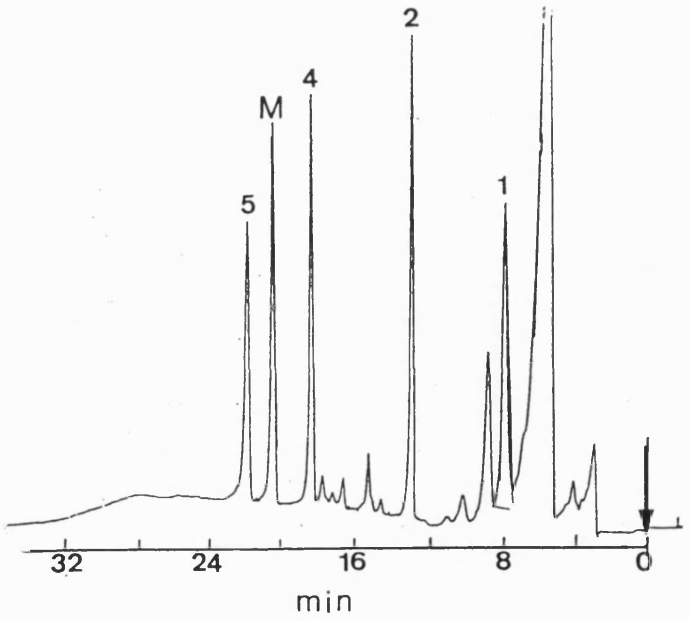


Fig 5.12 A sample chromatogram from the calibration plot on pkb-100 column using gradient elution (see page 153 for gradient conditions), alkylhalide derivatization using crown ether as a catalyst. (1) cocaine 0.76 μg ml^{-1}, (2) ethylcocaine 0.65 μg ml^{-1}, (4) butyl ester of BE 0.80 μg ml^{-1}, (M) methadone 0.75 μg ml^{-1}, (5) EME -TBDMS derivative 0.90 μg ml^{-1}.

B. MTBSTFA derivatization of ecgonine methyl ester

Initial attempts at EME derivatization with MTBSTFA proved successful using an acetonitrile solution and TBDMS derivatives were stable at room temperature for more than a week. Fig 5.13 shows a chromatogram obtained on the day of derivatization and the same sample injected a week later. Attempts to optimize the reaction condition proved frustrating as the results were not consistent. Increasing the amount of MTBSTFA from 50µl to 100 µl did not help.

MTBSTFA compared to other silylating agents is stable but is still hydrolysed by moisture present in the solvents, atmosphere etc. Silylation did not work when stock solutions were made up in methanol, evaporated to dryness and subjected to alkylation. Addition of acetonitrile to the dried residue and its subsequent removal under nitrogen was helpful, the reason being that acetonitrile forms azeotropes with water whereas other solvents used do not. Anhydrous conditions were crucial to the silylation step. Once the derivatives were formed as discussed above they were stable in aqueous solutions. Heating the vial at 60^0 C or 65^0 C for an hour or 45 minutes was found to be essential to give consistent results.

An attempt was made to carry out the alkylation and silylation reactions in a single step, but no conclusive results were obtained.

To establish the linearity of the method for the derivatization of EME as TBDMS derivatives a calibration was plotted using isocratic elution with a mobile phase of 28% ACN-0.05M KH_2PO_4 adjusted to pH 4.0 with 8.5% orthophosphoric acid. The calibration plot was linear in the concentration range examined 0.52µg to 2.90µg ml^{-1}. Fig 5.14 shows the calibration , intercept -0.01, slope 0.445, r= 0.993.

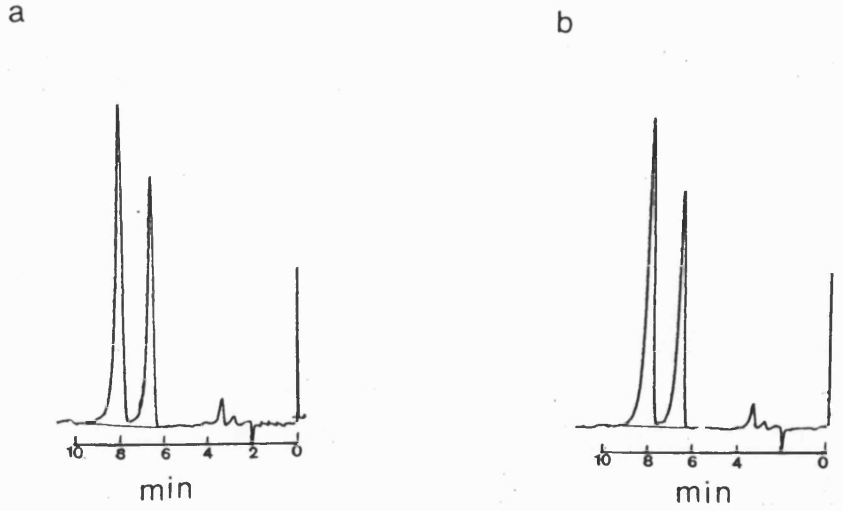


Fig 5.13 A Sample chromatogram showing stability of silyl derivative in aqueous solutions (a) on day one and (b) seven days later.

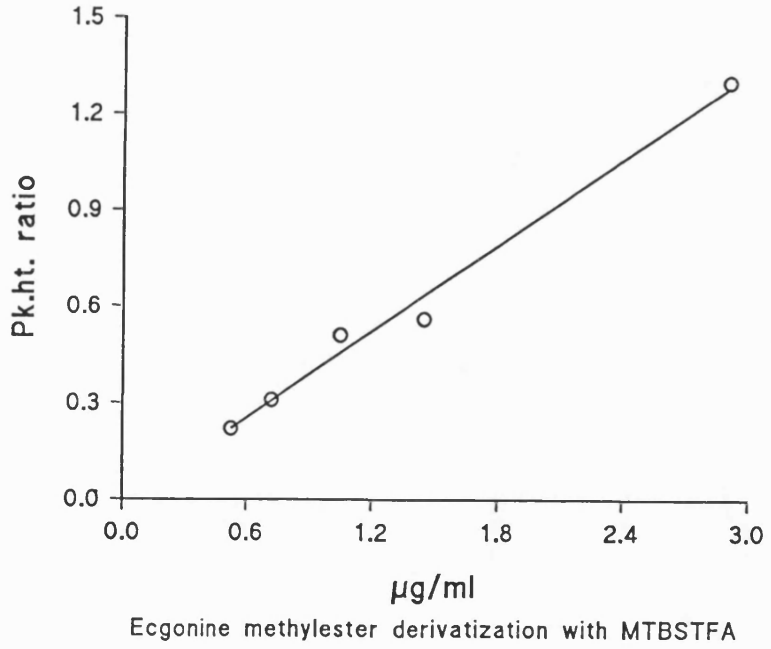


Fig 5.14 A calibration plot of EME derivatized with MTBSTFA, conditions as specified in the text.

Modified procedure for the derivatization of cocaine metabolites

Method B

(A) To the dried residue of analyte standards, 180µl of acetonitrile, 30µl of iodobutane, 10 mg of K_2CO_3 and 20 µl of 0.20 mg ml^{-1} of 18-Crown-6 ether in acetonitrile were added. The reacti-vial was closed and vortexed for 30 seconds, and then heated at 85° C for 1 hr. After the vials were allowed to cool, the samples were evaporated under N_2.

(B) Silylation of EME. Acetonitrile (100µl) and MTBSTFA (50µl) were added to the dried residue from (A) and the vials heated at 65° C for 45min. The samples were then allowed to cool and evaporated to dryness under a gentle stream of N_2. Mobile phase and internal standard were added to the dried residue and its pH adjusted with 8.5% phosphoric acid to that of the mobile phase. The samples were vortexed for 30 seconds injected into the HPLC system.

The modified procedure involved less sample manipulation as compared to method A described earlier thereby increasing the precision. However the presence of K_2CO_3 required the adjustment of pH prior to injection which might affect the column. Attempts to replace K_2CO_3 with a volatile organic amine such as triethylamine (TEA) were unsuccessful as TEA proved very difficult to remove and caused problems with the detectors.

Using the modified derivatization procedure the calibration curves were linear for cocaine and its metabolites in the range (µg ml^{-1})shown below. The equations for the calibration curves with standard deviations in brackets (±) and sensitivity limits for standard solutions were as follows:

Cocaine (0.95-0.095 µg ml^{-1}) y=0.910 (± 0.098) x + 0.05(±0.057); n=5; r=0.998; 0.06 µg ml^{-1}

Ethylcocaine (0.82-0.08µg ml^{-1})y=2.38 (± 0.324) x + 0.11(±0.16); n=5; r=0.997;

0.026 µg ml^{-1}.

Butyl ester of benzoylecgonine (1.02-0.102 μg ml^{-1}) y=1.20 (± 0.130) x + 0.138(±0.08); n=5; r=0.998; 0.08 μg ml^{-1}.

EME (TBDMS derivative)1.16-0.14 μg ml^{-1} ; y=1.35 (±0.080) x - 0.020 (±0.056); n = 5; 0.08 μg ml^{-1}.

5.9.3 Sample preparation

Liquid-liquid extraction and solid phase extraction have both been used for the extraction of cocaine and its metabolites from biological samples. LLE is quicker than solid phase extraction if small volumes of samples are involved, e.g 2ml but the clean-up and reproducibility is a problem. For LLE the general procedure is to add the sample in a test tube, adjust it to pH 9.0 and extract it in a mixture of chlorinated organic solvent and an alcohol by vortexing it for 1 minute, removing the upper aqueous layer and evaporating the organic solvent. If no derivatization is involved then the sample is dissolved in the mobile phase and injected on to the chromatographic system.

Solid phase extraction cartridges are gaining increasing popularity among various workers. Various speciality phases are available for drugs of abuse including Bond Elut Certify™ (Varian), Narc 2™ (J.T.Baker) and Clean Screen DAU ™ (World Wide Monitoring). Bond Elut Certify and Narc 2 are silica based. Bond Elut Certify is a mixture of C_{18} and cation exchange, Narc 2 is a mixture of cation exchange and cyano based material, Clean Screen is a copolymer with dual activity (cation exchange and non polar interactions). The exact composition is not disclosed. These cartridges are especially marketed for the extraction of drugs of abuse e.g cocaine and BE from urine and subsequent analysis by GC-MS. However, they have also been used for samples which have been analysed by HPLC (Browne et al 1992) or GC-NPD (Ortuno et al 1990).

The disadvantages of SPE are lack of reproducibility between various batches of cartridges, single use only, increased cost per sample and the need for special equipment. Advantages

are ease of automation which allows a large number of samples to be processed in one run around 24, often a better sample clean-up, and minimum amounts of organic solvents. Although the procedure may vary slightly for cocaine and metabolites among all the three manufacturers, the general procedure is:

1.The pH of the sample is adjusted between 4-6.

2. The cartridge is first conditioned with methanol followed by a buffer pH 6.0.

3. While the cartridge is still wet, the sample is applied on to the cartridge and pulled through at a very low flow rate (1 ml min^{-1}).

4. The cartridge is then washed with water, followed by an acid wash and then methanol.

5. The sample is then eluted with a mixture of 2-4% NH_4OH in dichloromethane-isopropanol (80-20 v/v).

The organic solvent is evaporated and the sample processed in the usual way.

In this work:

Sample: means Spiked sample and were prepared as follows :

Stock solution of cocaine and its metabolites was added to a test tube and evaporated to dryness using N_2. To the dried residue 1 ml of urine was added and the sample vortexed.

Blank: urine which did not contain any of the metabolite standards.

Control: metabolite standards spiked into water instead of urine.

Test: a cartridge to which no sample, control or blank was applied but treated as though it was, used to look for interfering peaks from the cartridges.

Initial attempts at sample preparation for cocaine and metabolites were made using LLE (Lau et al 1990). A stock solution of cocaine and metabolite standards was evaporated to dryness in a test tube . To the dried residue 1 ml of urine and 1 ml of buffer pH 9.2 was added and the mixture vortexed for 1 min. To this 5 ml of chloroform-ethanol (80:20 v/v) was added and the sample was vortexed for another 1 min. It was then centrifuged at 2000 rpm

for 5 minutes. The upper aqueous layer was removed and the organic layer evaporated to dryness and derivatized following Method A as discussed above in Section 5.9.2 F.

Blanks were prepared in the same way as the sample except that no stock solutions of standards were added.

The recoveries were calculated using the following equation:

$$\text{Percentage recovery} = \frac{\text{Pk.ht of extracted sample}}{\text{Pk.ht of non-extracted sample}} \times 100$$

The recoveries for cocaine and various metabolites were : Cocaine 91.8%, ethylcocaine 94.7%, norcocaine 120%, BE 50% and EME 80%. Although these recoveries look excellent, there were however interfering peaks under cocaine, ethylcocaine, norcocaine and EME as can be seen in Fig 5.15 (a) and (b).

The above procedure was repeated using a sample (spiked urine), a control and a blank. The extraction was performed using chloroform-isopropanol (80:20 v/v). The recoveries were as follows:

Metabolites	Control	Sample
Cocaine	100	116
Ethylcocaine	123	153
Norcocaine	78.34	89.90
BE	53.84	61.53
EME	70	93.53

The results from these experiments showed that the blank wasn't clean enough. Two approaches were considered.

1. Use of gradient elution so that the early eluting peaks can be separated from the matrix interferences.

2.Use of solid phase extraction cartridges.

A large number of urine components are either amphoteric like BE, or are secondary amines and these components remaining in the extract will be derivatized during the alkylation step used for derivatization of BE and norcocaine and will appear as interfering peaks. Solid phase extraction cartridges offered the advantages of a better clean up than LLE. Solid phase extraction work carried out in this chapter used Bond Elut Certify cartridges, unless otherwise stated.

Initial attempts using the manufacturer's procedure (Chapter 2) gave recoveries of 'control' of 90% for all cocaine metabolites except EME, which was 38%. Attempts to increase the recoveries by increasing the pH of the conditioning buffer did not help. This was also found by Ortuno et al. (1990) The blank was comparatively clean compared with LLE as can be seen from the chromatogram in Fig 5.16 (a) and (b).

To see whether the cartridges could be used again, a study was conducted to compare the recoveries of new cartridges with used ones, for control as well as sample following the manufacturers procedure.

Cartridge		Cocaine % recovery	EC % recovery	NC % recovery	BE % recovery	EME % recovery
New	Control	100	100	98.3	100	40
	Sample	94	99	91	90	38.93
Used	Control	73	60	60	45	10
	Sample	40.54	40.35	51.54	35	5

EC = ethylcocaine, NC = norcocaine, BE= benzoylecgonine, EME= ecgonine methyl esterderivative. After extraction samples were derivatized as described earlier

The results showed that cartridges could be used only once, as claimed by the manufacturer. The low recoveries were probably due to the irreversible binding of the endogenous components of the urine to the binding sites of the cartridge material.

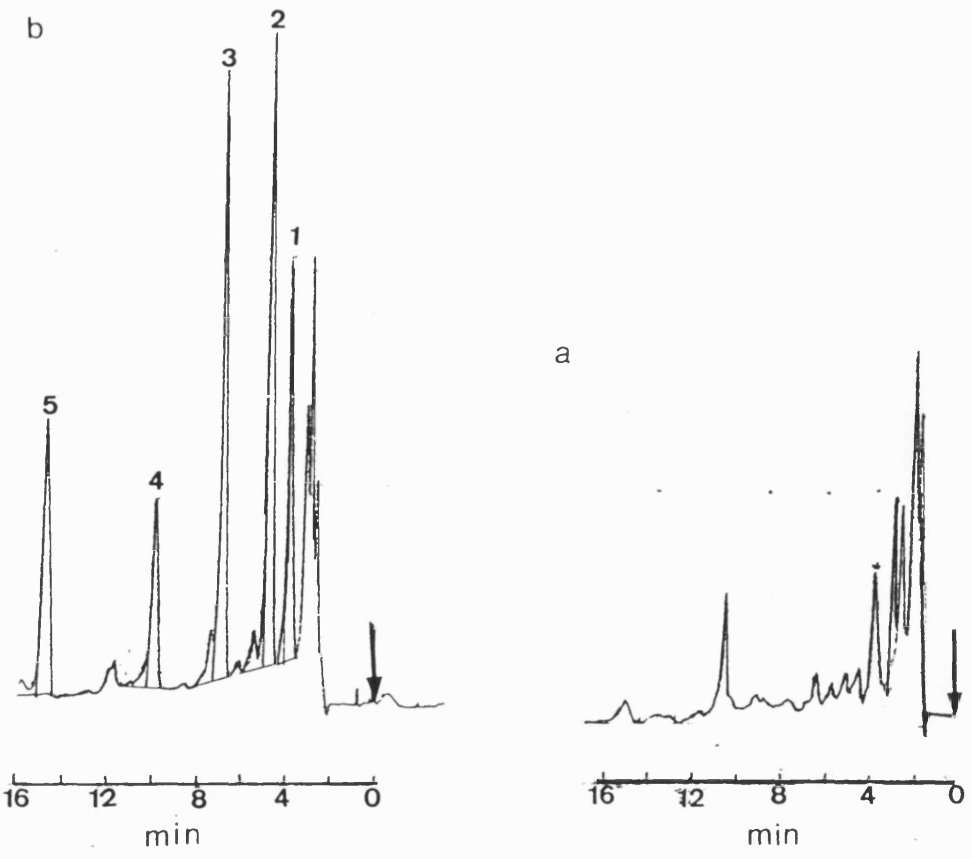


Fig 5.15 LLE of cocaine metabolites (a) blank urine, (b) spiked urine; column Supelco pKB-100

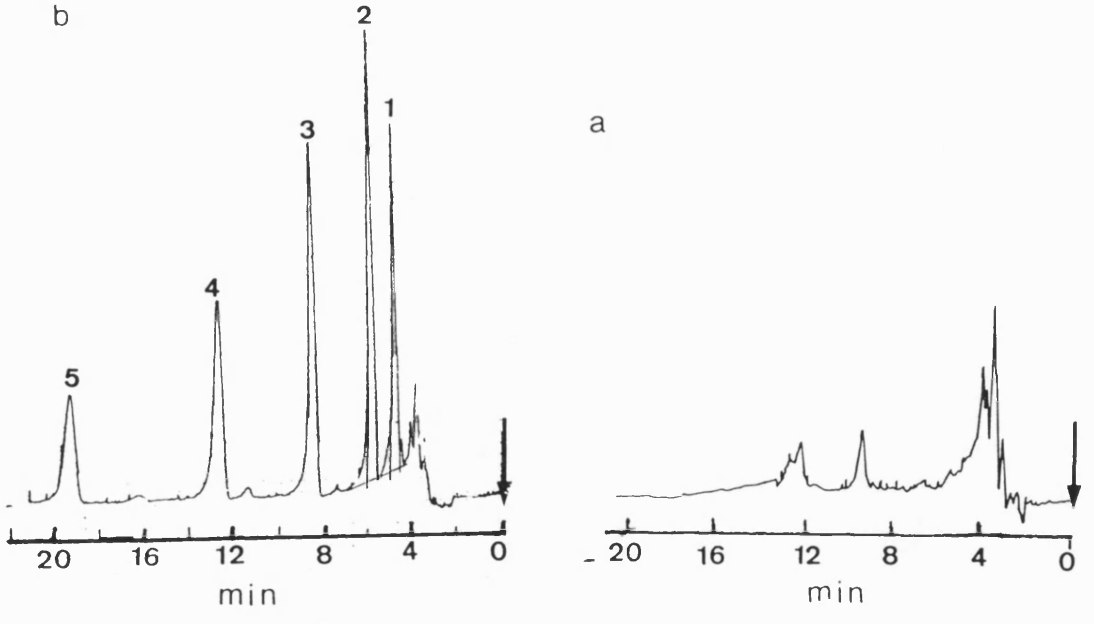


Fig 5.16 SPE of Cocaine metabolites (a) blank urine (b) spiked urine.

As the recoveries of EME were considerably less compared to the other metabolites, every stage of the sample preparation procedure was investigated using GC-MS. It was found that EME was being lost during the first washing step. EME is very water soluble and is very difficult to retain on the reversed phase material. To improve the recoveries of EME the cartridge capacity was increased.

Bond Elut Certify 300 mg cartridges

In the initial part of this work various batches of cartridges were tried as due to financial constraints only a small supply was available.

Initial results with the 300 mg cartridges (using the procedure for the 150mg cartridges) were very successful as recoveries in excess of 90% were obtained for all the metabolites. However this cartridge also retained endogenous material from the urine. Fig 5.17 shows a chromatogram of blank urine and spiked urine sample. The volume of eluting solvent was increased from 2 ml to 5 mls. To clean the urine blank more thoroughly the volume of conditioning and wash solvents was increased proportionally. The washing procedure used was:

Wash with 10 ml of buffer followed by 20 ml of 0.1N HCl, followed by 25 ml of methanol.

The sample was eluted with 5ml of 2% NH_4OH in DCM:IPA (80:20 v/v).

Fig 5.18 shows a chromatogram of blank urine and a control specimen.

The recoveries for the metabolites were as follows: Cocaine 100%, ethylcocaine 71%, Norcocaine 60%, BE 70% and EME 10%. The recoveries of the metabolites were approximately related to their hydrophobicity which meant that the volumes of buffer and methanol used were too great.

Marko et al. (1990) compared acetonitrile and methanol as eluting solvents for basic drugs. Using a C_{18} based cartridge, they showed that methanol was a good eluting solvent and

acetonitrile a good wash solvent regardless of the drug, support or concentration of hydrogen ions. The basic drugs (pentacaine, propanolol, stobadin) used in this study had a pKa close to cocaine and its metabolites (8.6 - 9.45), therefore acetonitrile was tested as a wash solvent.

The washing step involved washing with 4mls of conditioning buffer or water, followed by 8 ml of 0.1N HCL, followed by 10 ml of acetonitrile. The recoveries for control were: Cocaine 101%, ethylcocaine 94%, norcocaine 70%, benzoylecgonine 101% and EME 95%. Figure 5.19 shows chromatograms of control and blank under these conditions.

Repeating the above procedure with a different specimen of urine resulted in a more contaminated blank than was anticipated and the recoveries were also matrix dependent ranging from 50 to 60% for all the metabolites. To standardise matrix variations a commercial urine sample 'Lyphocheck' Urine Toxicology Control Negative (Bio-rad) was obtained for method development and was reconstituted with distilled water according to manufacturers instruction.

The washing procedure was slightly modified as follows:

Wash with 3 ml buffer pH 6.0 followed by 9 ml of 0.1 N HCl followed by 15 ml of acetonitrile. The recoveries for cocaine and metabolites were Cocaine 85%, ethylcocaine 70%, and 83% for norcocaine, BE and EME. Fig 5.20 shows a spiked urine sample.

New batches of cartridges (130 & 300 mg) showed extra peaks in the sample and blank. A thorough investigation of all the stages involved in the sample preparation step showed that these interfering peaks originated from the cartridge itself. These interfering peaks remained despite repeated washings with conditioning solvent i.e. methanol, acetonitrile, dichloromethane or the eluting solvent. Careful investigation revealed that these interfering peaks were related to the percentage of NH_4OH in the eluent as shown in Fig 5.21. Similar interferences were observed with the use of triethylamine as a 1% solution (no elution of

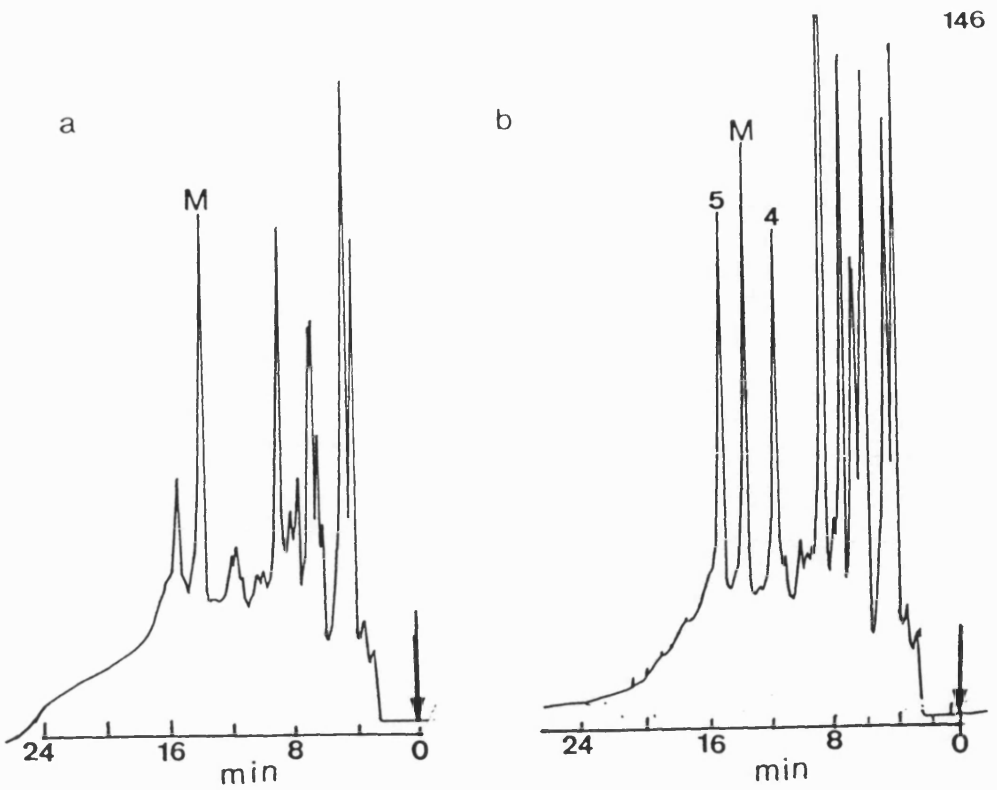


Fig 5.17 (a) blank urine (b) spiked urine, using Bond Elut 300 mg cartridges, conditions as specified in the text. (1) cocaine, (2) ethylcocaine, (3) butyl norcocaine (4) butyl BE, (M) methadone, used as internal standard, (5) EME-TBDMS derivative.

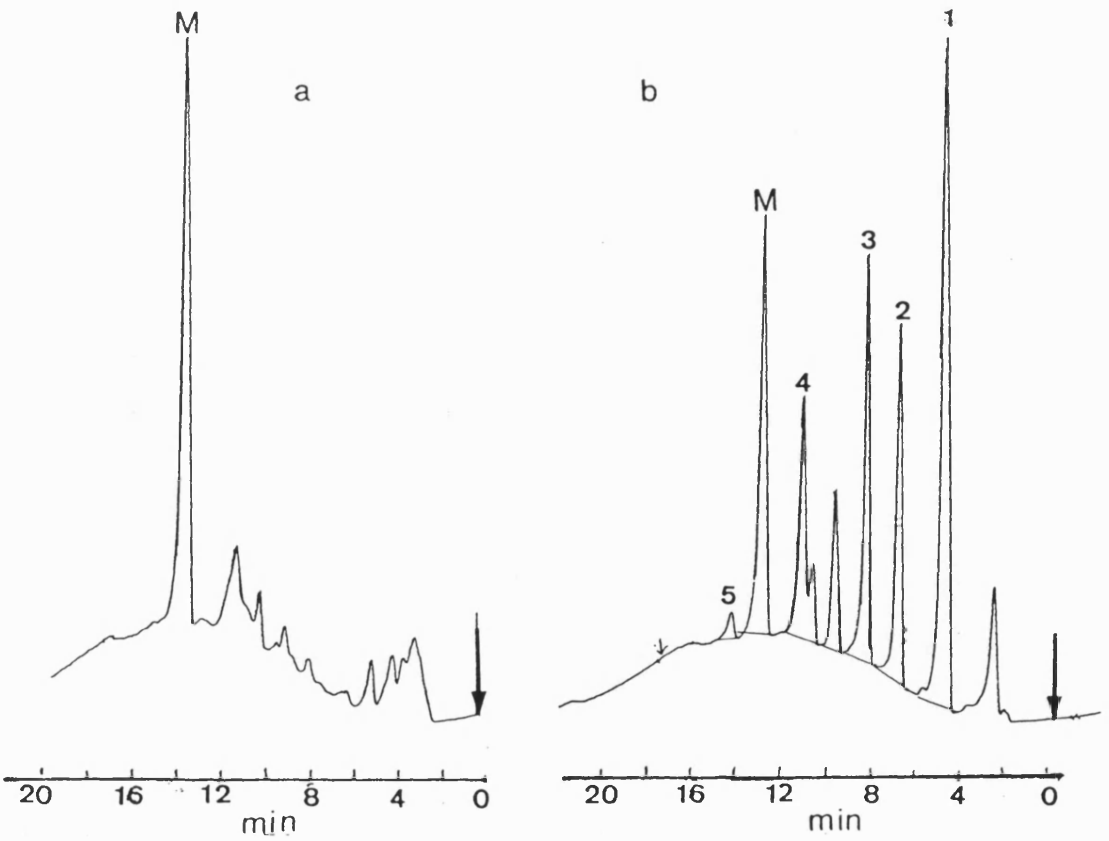


Fig 5.18 (a) blank (b) control, conditions as specified in the text.

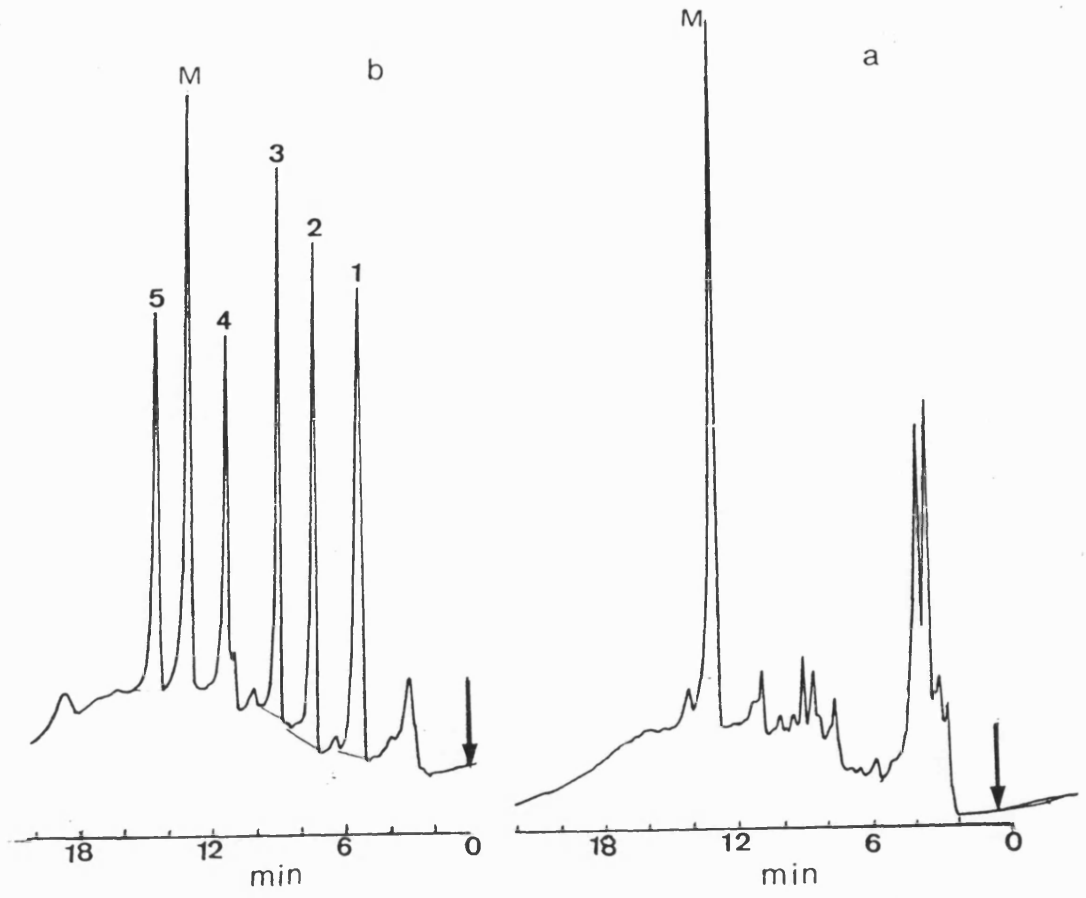


Fig 5.19 (a) blank (b) control, conditions as specified in the text.

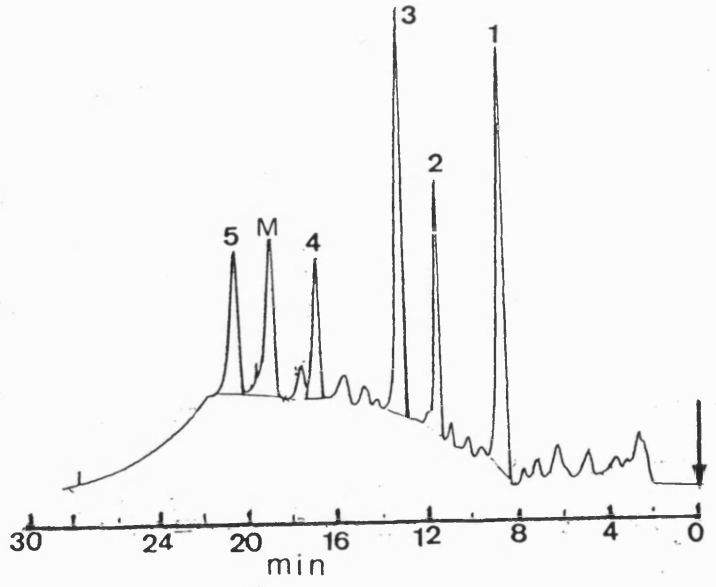


Fig 5.20 Sample chromatogram of a spiked urine sample using the modified washing procedure.

Fig 5.21 Bond Elute 'Certify' cartridge

Effect of elution step using ammonia in dichloromethane – IPA (80:20 v/v).

No standards or urine added to cartridge.

Methadone (M) added after elution step.

Residues derivatised by alkylation and silylation.

A 2% ammonia
B 1% ammonia
C 0.5% ammonia

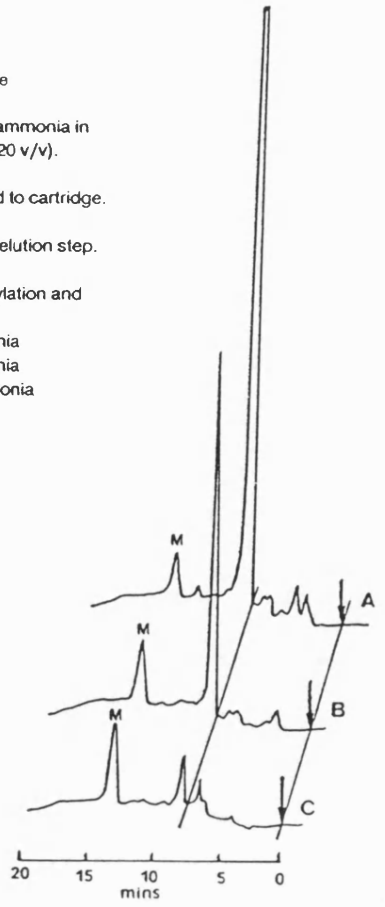


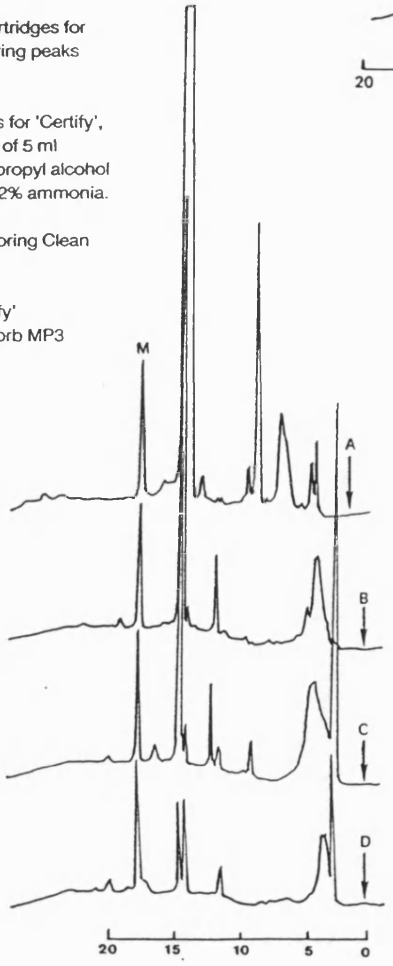
Fig 5.22

Comparison of SPE cartridges for the presence of interfering peaks in extract residues.

All cartridges treated as for 'Certify', with a final elution step of 5 ml dichloromethane – isopropyl alcohol (80:20 v/v) containing 2% ammonia.

A Worldwide Monitoring Clean Screen DAU.
B Baker Narc-2
C Bond Elute 'Certify'
D Interaction Polysorb MP3

M = methadone



BE). These interferences were present even in underivatized samples. An elution solvent containing 0.2% ammonia did not give any interfering peaks and gave a very clean blank with the wash procedure when used with the 130 mg cartridge. The recoveries of all the cocaine metabolites were above 80% except BE which did not elute at all. BE is the most basic cocaine metabolite (pKa 11.0) and also has an acidic group with a pKa of 2.0. Whilst the amount of ammonia used in the eluting solvent probably interacts with the free COOH group, this amount is probably insufficient to neutralize the charge on the SCX of the cartridge. The percentage recovery of BE increased with increasing percentage of ammonia in the eluting solvent. As a compromise 1.5% v/v ammonia in the eluting solvent was the least that could be used for good recoveries of all cocaine metabolites.

The presence of interfering peaks was also observed form the cartridges of other manufacturers as shown in Fig 5.22. The presence of interfering peaks were not observed in the initial stages of this work nor has this problem been reported by other workers who have used Bond Elut cartridges or Clean Screen DAU. It is very difficult to find an explanation for their occurrence. As these interfering peaks were related to the percentage of ammonia in the mobile phase a possible explanation could be that the ammonia is attacking the silica based packing material. The exact structural identification of these interferences was not possible even using GC-MS. However some generalizations can be made based upon their behaviour regarding the post-column ion-pair extraction system.

1. The substances appearing as interfering peaks are sufficiently hydrophobic to be retained for about 15 minutes on the column.

2. They have an ability to form ion-pairs with the fluorescent ion pairing agent.

As the initial cartridges were from mixed batches it was very difficult to find the batch history of earlier cartridges and compare them with the present ones. Following discussion with the manufacturers, improvements have been made in their cartridge material manufacture, although the new batch did not arrive in time for these studies.

The sample preparation which was finally used and gave a comparatively cleaner blank with a variety of urine samples was:

[1] Condition cartridge with : 3ml methanol

3ml of 0.1M phosphate buffer pH 6.0

[2] Sample: 1ml urine + 3(4) ml of phosphate buffer pH6.0

(the volume of buffer depends upon the consistency of the sample)

[3] Apply sample to the cartridge while it is still wet with the conditioning buffer, without vacuum. It is helpful to add 1 ml of conditioning buffer to the cartridge at this stage before sample is applied, one reason for low recoveries is the drying of the cartridge before the sample is applied.

[4] Washing steps 6 ml of distilled water

Air dry for 5 min at 15 mm (Hg)

9 ml 0.1M HCl

6 ml Methanol

3ml Acetonitrile

[5] Elution step: 5ml of dichloromethane:isopropanol (80:20 v/v) containing1.5% of NH_4OH.

[6] Evaporate to dryness (N_2)

[7} Alkylation step (Method A or Method B)

[8} Silylation step

[9] Evaporate to dryness (N_2)

[10] Add internal standard and make up the volume in 0.5 ml of mobile phase.

Fig 5.23 (a), (b)shows a chromatogram of blank urine and spiked urine using the above procedure. Fig 5.24 shows a chromatogram of metabolite standards. Method A was used for alkylation.

Internal standard

An internal standard (I.S) is a substance that is added, in known concentrations, to the sample at the earliest possible point in an analytical scheme to compensate for sample losses, occurring during sample clean-up, derivatization and final chromatographic analysis.

Internal standards can be classified as: (a). recovery standards, to check for the sample-clean up, (b). derivatization standards, to check for derivatization efficiency, and (c). chromatographic standards, to compensate for instrument problems i.e. sample size, gradient elution, depending upon the point where they are added to the sample. This classification is however arbitrary. It is best to have an internal standard which can act as all three at the same time

An ideal internal standard should :

1. be structurally similar to analyte (s)

2. undergo same reactions as the analytes

3. elute within a reasonable length of time

4. the internal standard and the analytes should be baseline resolved.

5. not be present in the original sample

6. can form ion-pairs with fluorescent counter-ion DAS using post-column conditions.

Unfortunately no substance was found which could meet all these criteria, or independently act as recovery or derivatization standards without complicating the analytical method

requirements. However various substances were synthesized and tested as prospective internal standards. These substances in this study were used as chromatographic standards but can also be used as recovery standards if needed. As these substances were tested under a variety of gradient conditions together with standards, spiked urine and blanks, the retention times have been adjusted to the chromatographic conditions in Fig 5.23 (b).

Propylbenzoylecgonine	PrBE	12.8 min
Pentylnorcocaine	PNc	15.0 min
Pentylbenzoylecgonine	PBE	20.0 min
Hexylbenzoylecgonine	HBE	27.2 min
Methadone	M	18.6 min

The resolution between PrBE and Norcocaine was 0.8 and the resolution could not be improved between the two by changing the gradient without significantly affecting the chromatography of late eluting peaks. Moreover the interferences from sample components were also present in this area.

PNc eluted at the same time as the main interfering peak from the cartridge and the two could not be separated. The retention time of PBE and EME t-BDMS derivative were too close.

Because of the above reasons most of them were not suitable under the conditions used for the analysis of cocaine and metabolites.

Methadone and HBE eluted at points in the chromatogram which were free from other analytes or interfering peaks. HBE eluted at around 27.2 minutes which added another 8 minutes to the analysis time, (Fig 5.23, b), thus increasing the time between injections from 40 minutes to 60 minutes. Methadone was found to be the most suitable for practical purposes. Methadone was added to the samples before injection to check for variations in

the post-column ion-pair extraction system. Routinely, the use of methadone may be a disadvantage as it is used as a drug to wean patients from heroin and may be present in some cocaine users, and in such cases HBE would be more suitable as an internal standard at the expense of a longer analysis time.

The variations in retention time between various chromatograms is because various gradient conditions were tried before the best were found which could be used for standards as well as urine samples.

The calibrations were prepared using the mobile phase at 0.4ml.min^{-1} consisting of a step gradient at 7 minutes from 11 to 30% v/v acetonitrile in 0.05M KH_2PO_4 adjusted to pH 4.0 with 8.5% orthophosphoric acid and then returned to 11% at 25min. The samples were derivatized according to Method B as described earlier (Section 5.9.3).

Peak height ratios were measured and plotted against concentration of standards. The recoveries of sample from urine were calculated by comparison with non-extracted standards. Fig 5.25 shows sample chromatograms of blank urine (a), spiked urine (b). The calibration curves were linear from 200 to 1000 ng ml^{-1} for all metabolites. The equations for the calibration curves for extracted samples with standard deviations in brackets (±) were as follows:

Cocaine y= 0.913 (±0.142) x+ 0.001(± 0.081); n=4 ;r =0.999 ;

LOD 0.059 µg/ml.

Ethylcocaine y= 2.68 (±0.712).x + 0.097 (± 0.395) ;n=4 ;r= 0.995 ;

LOD 0.095. µg/ml.

BE y=1.61 (±0.165) x - 0.126 (±0.114) ;n=4 ;r= 0.999 ;

LOD 0.046 µg/ml.

EME y= 0.983 (± 0.156) x - 0.021(± 0.123) ;n=4 ; r= 0.998 ;

LOD 0.081 µg/ml.

LOD = limit of detection (Miller and Miller, 1988)

Recoveries were as follows:

Compounds	High	Low
	1000 ng ml	200 ng ml
Cocaine	96	70
Ethylcocaine	80	100
BE	84	91
EME	84	91

The recoveries of BE were higher because it was close to the interfering peaks.

Validation

Within-day precision was calculated by repeating a medium point from the calibration point and extracting it the same day using four different cartridges

Within day precision ±s.d for (n=4) were as follows:

Analytes	Standard Pk ht ratio	Sample Pk ht ratio (± sd)	Pecent recovery
Cocaine	0.79	0.78 (0.056)	100
Ethylcocaine	1.20	0.96 (0.047)	80
butyl-BE	1.05	0.96 (0.045)	91
EME (TBDMS deriv)	0.575	0.56 (0.037)	98

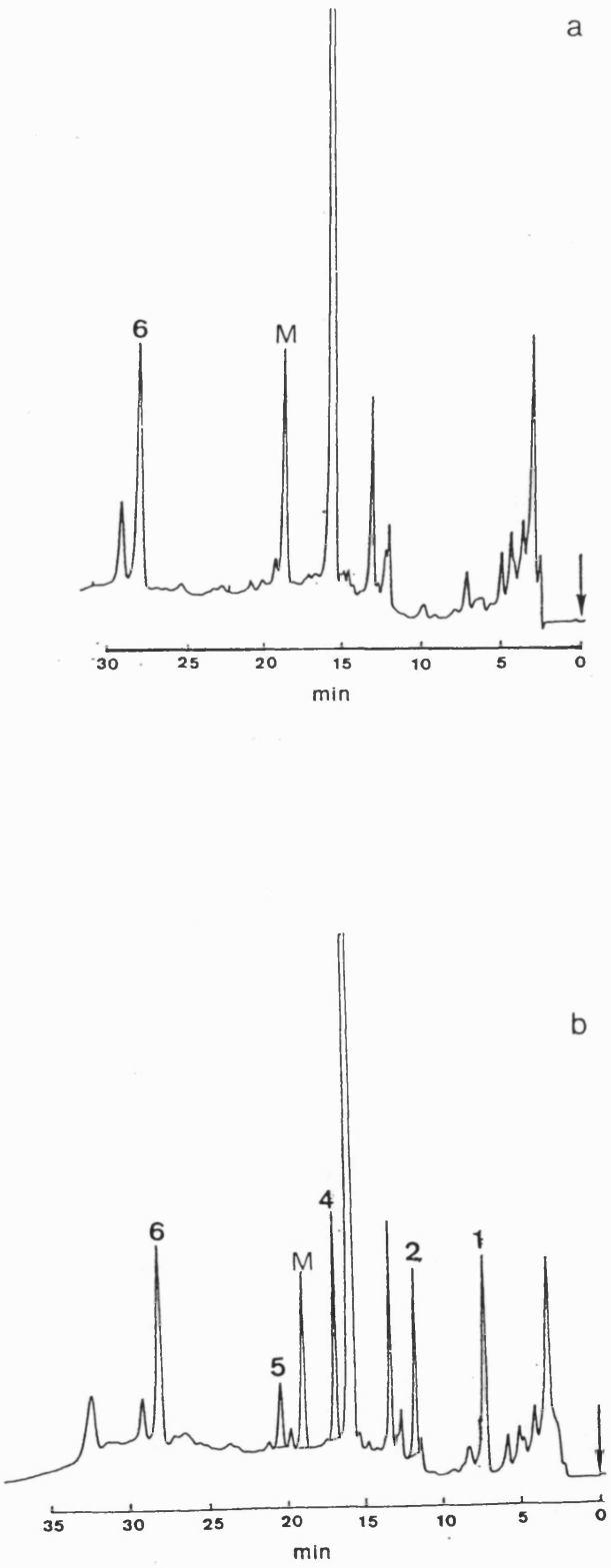


Fig 5.23 (a) blank urine, (b) spiked urine, conditions as specified in the text. (Method A for alkylation)
(1) cocaine 0.6 μg ml^{-1}, (2) ethylcocaine 0.44 μg ml^{-1} , (4) BE 0.5 μg ml^{-1}, (M) methadone 0.30 μg ml^{-1}, (5) EME 0.6 μg ml^{-1}, (6) hexylbenzoylecgonine 2.0 μg ml^{-1}.

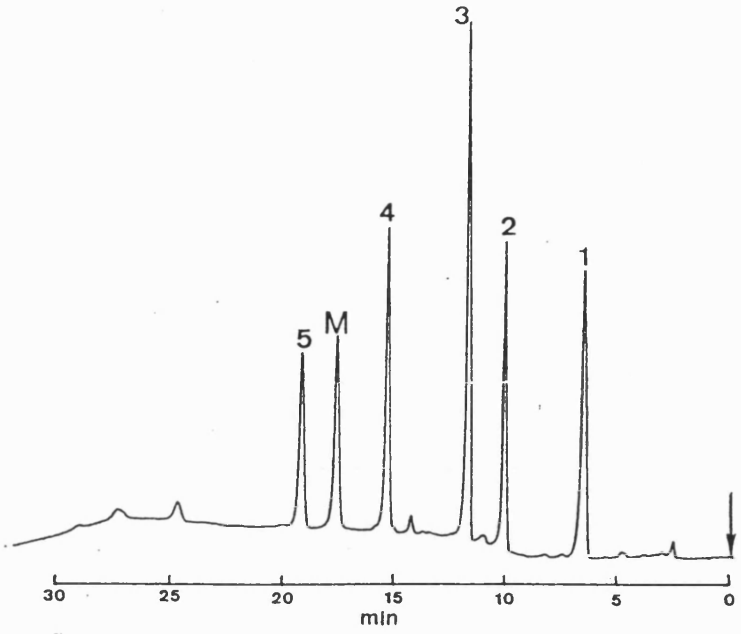


Fig 5.24 A sample chromatogram of metabolite standards, conditions as for Fig 5.23. (1) cocaine 0.6 μg ml^{-1}, (2) ethylcocaine 0.44 μg ml^{-1}, (3) N-butylnorcocaine 0.4 μg ml^{-1} (4) BE 0.5 μg ml^{-1}, (M) methadone 0.30 μg ml^{-1}, (5) EME 0.6 μg ml^{-1}.

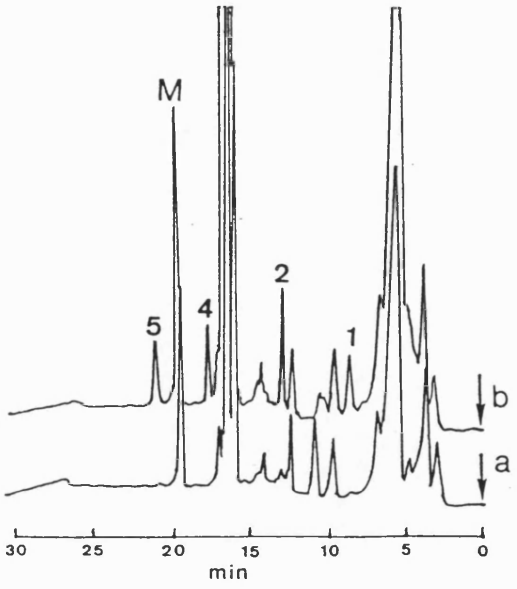


Fig 5.25 A sample chromatogram from the calibration curve (a) blank urine (b) spike urine . (1) cocaine 0.0.23 μg ml^{-1}, (2) ethylcocaine 0.16 μg ml^{-1}, (4) BE 0.20 μg ml^{-1}, (M) methadone 0.75 μg ml^{-1}, (5) EME 0.20 μg ml^{-1}.

Specificity

A limited number of drugs of abuse were tested for their behaviour on the cocaine system to give some indications of the specificity of the procedure.

Compound	t_r	Compound	t_r
Cocaine	7.8 min	Normethadone	16.8
Pethidine	9.2	BE	18.2
Pipradol	11.8	Methadone	19.80
Ethylcocaine	13.0	EME	21.7
Benzphetamine	14.0	Dipipanone	23.4
Fentanyl	15.0	*****	****

5.10 Conclusions

In this chapter the use of iodobutane and silylation as precolumn, non-labelling derivatization techniques has been demonstrated to improve the chromatographic properties and enhance detection sensitivities of analytes especially benzoylecgonine and EME.

For aqueous standards the lowest point on the calibration was 100 ng ml^{-1} for all the cocaine metabolites, however due to the presence of interfering peaks from the cartridges 200 ng. ml^{-1} of cocaine and its metabolites was easily detectable as can be seen from Fig 5.25 (b). The sensitivity achieved for BE (100 ng ml^{-1}) is good enough for confirmation of cocaine abuse as required by regulatory agencies like NIDA (see page 99). This increase in sensitivity is comparable or even slightly better than some other methods e.g Ortuno et al 1990, who achieved a sensitivity limit of 100 ng ml^{-1} for cocaine and BE and 250 ng ml^{-1} for EME , and 100 ng ml^{-1} for cocaine and its metabolites (Miller and DeVane 1991).

This work also demonstrates the suitability of MTBSTFA as a silylating agent for the derivatization of hydroxyl groups for HPLC. Previously this reagent has only been used for gas chromatographic analysis.

The use of 18 Crown 6 in combination with K_2CO_3 has reduced the derivatization time from 3 hrs (Ortuno et al) to 1 hr.

The use of larger capacity cartridges (300 mg) has improved the recovery of EME from 40% to above 80%.

The Suplex pKb 100 column was found to be good in terms of chromatographic properties for basic drugs and has a long column life as compared with Cyano columns which were used at the beginning of this work.

On the negative side, the presence of K_2CO_3 requires the adjustment of pH which if not done correctly adversely affects the column. No suitable volatile organic base was found to overcome this problem.

A 250 mm column, although efficient gave long analytical times, as the time between injections was a minimum of 45 minutes. A shorter column would cut the analysis time considerably.

Sample preparation in this work proved very frustrating because of the presence of interfering peaks. Perhaps cartridges which are not silica based e.g. Polysorb MP3 may be the answer or the new batch of Bond Elut certify cartridges may offer some improvement.

Although in this work pre-column derivatization has been used in combination with post-column ion-pair extraction detection, it is appreciated that many laboratories will not have the necessary facilities or the technical know-how required for the post-column work. Goto et al (1983) and Kubo (1986) have demonstrated the use of 1-anthronyl nitrile for the derivatization of hydroxy groups of prostaglandins and steroids. The same reagent can also be used for the pre-column derivatization of EME without the use of post-column ion-pair extraction set-up.

6 QUATERNARY AMMONIUM COMPOUNDS

6.1 Introduction

Quaternary ammonium compounds readily form ion-pairs with negatively charged ion-pairing agents and so can serve as useful agents for the testing of post-column ion-pair extraction detectors. In order to demonstrate the usefulness of post-column ion-pair extraction detectors to real analytical problems some pharmacologically active quaternary ammonium compounds were chosen to examine whether improvements could be made over existing methods in terms of sensitivity and selectivity.

The reversed phase chromatography of these compounds is problematic as they elute as badly tailing peaks when silica based reversed phase columns are used, (Liedekerke et al. 1989) even when silanol masking agents such as TEA or ion-pairing agents have been added to the mobile phase. It was therefore the aim of this study to develop a suitable chromatographic system so that these compounds would be eluted as sharp symmetrical peaks if possible .

The pharmacologically active quaternary ammonium compounds chosen for this study were edrophonium, neostigmine, pyridostigmine and the hydroxy metabolites of neostigmine and pyridostigmine. Apart from pyridostigmine ($\varepsilon < 2500$) they have low molar absorptivity $\varepsilon < 400$ at 254 nm and can therefore only be detected at low U.V.(214nm), which can increase sensitivity but is not selective enough. Fig 6.1 shows a published chromatogram obtained at this wavelength. All of these substances are anti-cholinesterases and are used in anaesthesiology to reverse the action of non-depolarizing neuromuscular blockade, or in myasthenia gravis. Pretreatment with pyridostigmine is used as an antidote to organophosphorus poisoning (nerve gas) e.g soman. (Gordon et al. 1978).

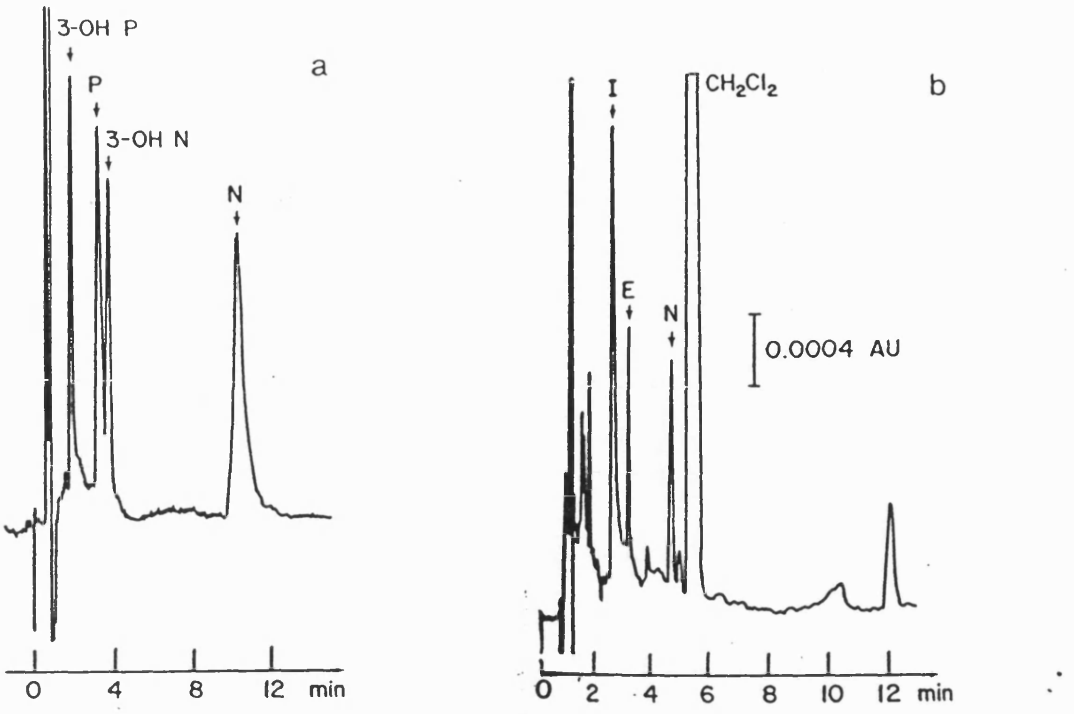
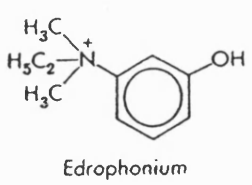
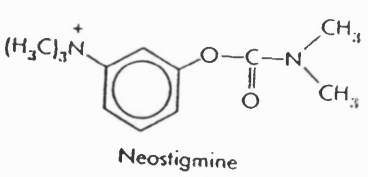


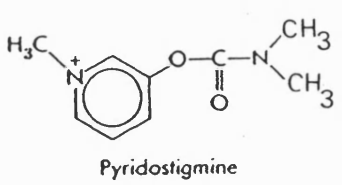
Fig 6.1 Chromatogram showing presence of interfering peaks (a) standard (b) serum extract (E) edrophonium (N) neostigmine (P) pyridostigmine (3-OH) hydroxy metabolites (Ruyter et al, 1980)



Edrophonium



Neostigmine



Pyridostigmine

Fig 6.2 Structures of quaternary ammonium compounds studied

The pharmacological effects of these drugs are strongly related to their plasma concentrations, and show a large variation between individuals, especially with pyridostigmine (Clavey and Chan, 1977). Excessive dosage of these drugs therefore can impair neuromuscular transmission and precipitate "cholinergic crises" by causing a depolarising block which is difficult to distinguish from a worsening myasthenic state (BNF p344 March 1991). For this reason it is important to develop sensitive methods for their detection.

6.2 Physicochemical properties

EDROPHONIUM CHLORIDE (Tensilon™)

$C_{10}H_{16}ClNO$, M.wt = 201.7 (Clark)

A white crystalline powder. Mpt 165° to 170°, with decomposition.

Soluble 1 in 0.5 of water and 1 in 5 of ethanol, practically insoluble in chloroform.

$A^{1\%}_{1cm}$ 110 at 273 nm.

NEOSTIGMINE BROMIDE (Prostigmin(e)™).

$C_{12}H_{19}BrN_2O_2$, M.wt = 303.2

Colourless crystals or white crystalline slightly hygroscopic powder Mpt 171°-176° with decomposition.

Soluble 1in 0.5 ml. of water, 1 in 8 of ethanol, 1 in 5 of $CHCl_3$ practically insoluble in ether.

$A^{1\%}_{1cm}$ 16 at 260 nm.

PYRIDOSTIGMINE (Mestinon)™

$C_9H_{13}BrN_2O_2$, M. wt = 261.6

A white deliquescent , crystalline powder Mpt. 153° - 157°.

Soluble 1 in less than 1 of water, 1 in less than 1 of ethanol, 1 in 1 of $CHCl_3$, practically insoluble in ether. $A^{1\%}_{1cm}$ 186 at 270 nm.

Fig 6.2 shows the structures of these quaternary ammonium compounds.

6.3 Pharmacology (Taylor, 1990)

Drugs that inhibit the acetylcholinesterase enzyme are called anticholinesterases and cause acetylcholine to remain at cholinergic receptor sites and produce effects which result from excessive stimulation of cholinergic receptors throughout the central and peripheral nervous system. Apart from their use in medicine they have found excessive use as toxic agents e.g as insecticides and nerve agents.. The anticholinesterases fall into three main groups according to the duration of action:

1) Short acting

2) Medium acting

3) Irreversible cholinesterase.

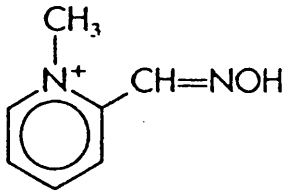
To understand how these drugs act a brief description is given of acetylcholinesterase.

The active sites of acetylcholinesterase consist of a negative subsite (glutamic acid), which attracts the quaternary group of choline through coulombic and hydrophobic forces, and an esteratic subsite (serine-OH) where in simple terms, acetylcholine is hydrolysed into choline. Acetylcholinesterase is capable of hydrolysing about 3×10^5 acetylcholine molecules per molecule of enzyme per minute.

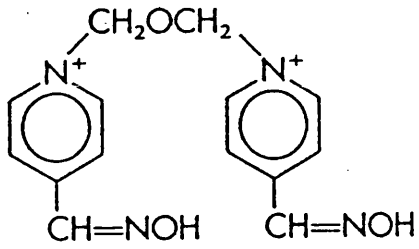
Edrophonium is a short acting drug and binds only to the anionic site (glutamic residue) of the enzyme. The ionic bond is readily reversible and because of rapid elimination of the drug the action of the drug is very brief. It is used mainly for diagnostic purposes, an improvement of muscle strength following treatment with an anticholinesterase is characteristic of the condition myasthenia gravis, and does not occur when muscle weakness is due to other causes.

Neostigmine, physostigmine and pyridostigmine are medium duration anticholinesterases. Like edrophonium they also bind to the anionic site of the enzyme and serve as an alternative substrate for enzymes. As they are carbamyl esters they are more resistant to hydrolysis than acetylcholine. The duration of inhibition by the carbamoylating agents is 3 to 4 hrs.

Organophosphorus compounds e.g sarin, soman, malathion are pentavalent phosphorus compounds which form stable irreversible complexes with the enzyme and the return of enzyme activity depends upon the synthesis of new enzymes. However the enzymes can be reactivated by the use of nucleophilic agents like hydroxylamine (NH_2OH) and oximes (RCH=NOH). Pralidoxime and obidoxime are more potent as activators and are used as antidotes for nerve agent poisoning.



Pralidoxime



Obidoxime

Apart from their use in myasthenia gravis, they are used (pyridostigmine) in the treatment of paralytic ileus and post-operative urinary retention. Pretreatment with carbamoyl esters is used as an antidote to organophosphorus poisoning (Dirnhuber et al.1979). The protective action of carbamates against organophosphorus poisoning depends upon the ability of carbamates to form a semi-stable complex with the enzyme, thus protecting the enzyme against phosphorylation, which can then break down to liberate the enzyme.

6.4 Pharmacokinetics

Edrophonium has a half life of 0.5 hrs. After I.V injection of 100 μg kg^{-1} to five subjects plasma concentrations ranged from 0.8-3.9 μg.ml^{-1} at 2 min. and declined to 0.038 -0.056 μg.ml^{-1}at 1 hr.(Clark 1986).

Neostigmine is given orally, but it is poorly absorbed after oral administration. It is metabolized by ester hydrolysis to form 3-hydroxytrimethylanilinium bromide, which is active. About 20 % of an oral dose is excreted in the urine as unchanged drug; 50% of oral dose is

eliminated in the faeces. After intamuscular (IM) injection 80% is excreted within 24 hrs, 50% as unchanged drug and 15% as metabolites (Clark, 1986).

After a single dose of 30 mg orally to 3 subjects, peak plasma concentrations of 0.004-0.009 $\mu g\ ml^{-1}$ were attained in 1 to 2 hrs (Aquilonius et al. 1980). After IM injection of 5mg to 5 subjects, plasma concentrations ranged from 0.84-6.25 $\mu g\ ml^{-1}$ at 2 min. Half life is about 1 hr, plasma clearance 11ml $min^{-1}kg^{-1}$(Clark).

Pyridostigmine is poorly absorbed from the gastrointestinal tract. It is excreted mainly in the urine as unchanged (16%) drug and small amounts of 3-hydroxy N-methyl pyridinium. After I.V administration 90% of the drug is excreted as unchanged drug in the urine.

In plasma, therapeutic concentrations are around 0.05 to 1.0 $\mu g\ ml^{-1}$. After a single oral dose of 120mg to 5 subjects peak plasma levels of 0.04-0.07 $\mu g\ ml^{-1}$ were attained in 1-2 hrs but showed considerable inter-individual variations (Calvey and Chan 1977). Plasma half life is 0.3 to 2 hrs, but is increased in subjects with renal disease.

6.5 Literature review of analytical methods

Edrophonium has been measured by an enzymatic assay that measures the activity of enzyme cholinesterase and is based on the principle that edrophonium combines reversibly with cholinesterase enzyme so that the inhibition of enzyme activity is related to the plasma concentration. A linear relationship was found between the plasma concentration of the drug and enzyme activity in the range of 0.1 to 2.0 $nmol.ml^{-1}$ (0.02-0.4 $\mu g\ ml^{-1}$) (Barber et al 1976). Although the method is quite sensitive according to Chan et al. (1976) it is quite tedious and time consuming.

A spectrophotometric method (Cooper et al, 1974) and a RIA (Radio-immunoassay) for pyridostigmine have been reported. The spectrophotometric method measures the drug as an iodide-ion-pair. The sensitivity of the method is 0.1– 0.2 $\mu g\ ml^{-1}$, which is not sufficient to measure pyridostigmine in plasma after administration to man of 30 to 60 mg doses (Ellin et

al. 1982). The RIA assay was able to measure 0.1 ng. ml^{-1} in 0.1 ml of plasma, the method was used for small samples e.g from rats. (Meyer 1988). Although the radioactive methods are quite sensitive, their use requires a special licence and there are problems with waste disposal.

Chan et al (1976) achieved a sensitivity limit of 5 ng ml^{-1} for neostigmine and pyridostigmine, using gas chromatography with a sample volume of 3 ml. The analytes were extracted from plasma as iodide ion-pairs into dichloromethane. The GC method depends upon thermal dequaternisation of quaternary ammonium compounds. The method was however not suitable for edrophonium because of its decomposition. Although the authors reported excellent reproducibility and ease of use as compared to above methods, in practice (Jennings, 1980) it requires special equipment, is plagued with secondary reactions and the reproducibility is not always good. Moreover the ionic nature of these compounds and their low volatility makes their gas chromatography difficult.

The published HPLC methods for these compounds include that of Ruyter et al. (1980) where all three compounds i.e., edrophonium, neostigmine and pyridostigmine were extracted from serum as picrate ion-pairs into dichloromethane and then chromatographed on a C_8 column. Two separate mobile phases were used, one for neostigmine and edrophonium containing heptanesulfonic acid and TMA^+Cl^- (tetramethylammonium chloride) in acetonitrile-water (20-80v/v) and the other for pyridostigmine which was acetonitrile-water (17-83 v/v) at pH 3.0; detection was at 214 nm. The authors reported the presence of interfering peaks from the sample which sometimes interfered with pyridostigmine (Fig 6.1). Quantification down to 5 ng. ml^{-1} (S/N > 4) was reported for each of the analytes. The metabolites of pyridostigmine and neostigmine were not quantified. Recoveries were edrophonium 87%, pyridostigmine 96% and neostigmine 100%. Liedekerke et. al, (1989) demonstrated the use of a polystyrene divinyl-benzene column for the chromatography of quaternary ammonium compounds. They chromatographed a series of compounds using water-tetra-methylammonium

hydroxide (TMAH) mobile phases with various percentages of acetonitrile adjusted to pH 2.0 and showed that pyridinium type compounds e.g pyridostigmine gave lowest values (1.0) for asymmetry (A_S) and highest values (1767) for column efficiency (N) as compared to N-phenyl-N-trialkyl type e.g edrophonium and neostigmine (A_S1.7 and N=1205 for edrophonium) which gave intermediate results, whereas aliphatic compounds e.g mepenzolate gave low theoretical plate counts (A_S1.3 and N =757).

Compared to edrophonium and neostigmine, more interest has been shown in developing sensitive methods for pyridostigmine probably because of its wide-spread use for myasthenia gravis and also as an antidote to nerve agent poisoning.

Yakatan and Tien (1979) reported an HPLC method for the determination of pyridostigmine using neostigmine as an internal standard. The drug was extracted from plasma, which had previously been washed with acid and methylene chloride, as perchlorate ion-pairs and chromatographed on an ODS column using an ACN-sodium lauryl sulphate - acetic acid mobile phase and detection at 269 nm. The authors reported the presence of interfering peaks (in aqueous and plasma samples), at the elution time of pyridostigmine which were attributed to an impurity present in the neostigmine. A detection limit of 20ng ml^{-1} was achieved using 1 ml of plasma.

Ellin et al. (1982) reported a similar HPLC method for pyridostigmine but used SepPak C_{18} cartridges at pH 10.0 for preparation of samples from urine and plasma. Recoveries were 90-95% using 5 ml of sample, a detection limit of 40ng ml^{-1} was obtained.

Matsunaga et al. (1987) reported a similar method for plasma but used Bond Elut CBA cartridges with chromatography on a cyano column using 0.1%TEA in water and acetonitrile, and reported recoveries of 95% with a limit of detection of 10 ng ml^{-1}. Michaelis (1990) combined the extraction procedure of Ellin et al. (1982) and chromatographic system of Matsunaga et al (1987) and reported a detection limit of 1-2

ng ml^{-1} using 2 ml of plasma, and tangent skimming as shown in Fig 6.3 taken from the above paper.

Malcom et al. (1990) reported a thermospray LC-MS for the measurement of pyridostigmine in plasma. According to the authors the method was capable of measuring plasma concentrations down to 1 ng ml^{-1} with chromatography on a silica based column. The plasma samples were extracted using an AASP C_8 cartridge. Although LC-MS is suitable for compounds which have very poor U.V detectability, unfortunately it remains a relatively expensive technique to purchase and maintain in good working order.

6.6 Synthesis of metabolites

6.6.1 3-OH TRIMETHYLANILINIUM BROMIDE (OHMN) (Ruyter et al. 1980)

Neostigmine bromide 2.0 gm. was hydrolysed by stirring with 2N sodium hydroxide at 50° C for 4 hrs. After completion of hydrolysis the solution was neutralised with conc HCl and freeze dried. The powder was dissolved in MeOH and filtered. The filtrate was evaporated to dryness using a Rotary evaporator and 200 mg of white crystals were obtained. The structure was confirmed by mass spectrometry (Fig 6.4 a) and ^1H N.M.R. The compound was found to be 99% pure by HPLC.

3.6.2. 3-HYDROXY N-METHYL PYRIDINIUM BROMIDE.

Pyridostigmine (200 mg) was stirred together with 10 ml of 2N sodium hydroxide in a 50 ml round bottom flask for 4 hrs at 50° C. After completion of the hydrolysis step the solution was neutralised with conc HCl and freeze-dried. The resulting powder was dissolved in methanol and filtered. The filtrate was evaporated to dryness, but the yield of powder obtained was negligible.

An attempt was then made to prepare the metabolite synthetically according to the method of Shapiro (1959).

A mixture of 4.75 gm of 3-hydroxy pyridine and 3.11 ml of methyl iodide in 25 ml of 1-propanol was heated under reflux for 8 hrs. After removal of propanol the residue was left in a vacuum oven to dry to a constant weight to give 6.75 gm of brown coloured granules. The structure of the compound was confirmed by mass spectrometry (Fig 6.4 b) and ^{1}H-NMR. TLC of the sample on a silica gel plate with 100% ACN gave two spots when viewed under U.V light at 254 nm. The product was purified by repeated crystallisation with an acetone-ether mixture until a single spot was obtained. About 1.0 gm of cream coloured crystals were obtained.

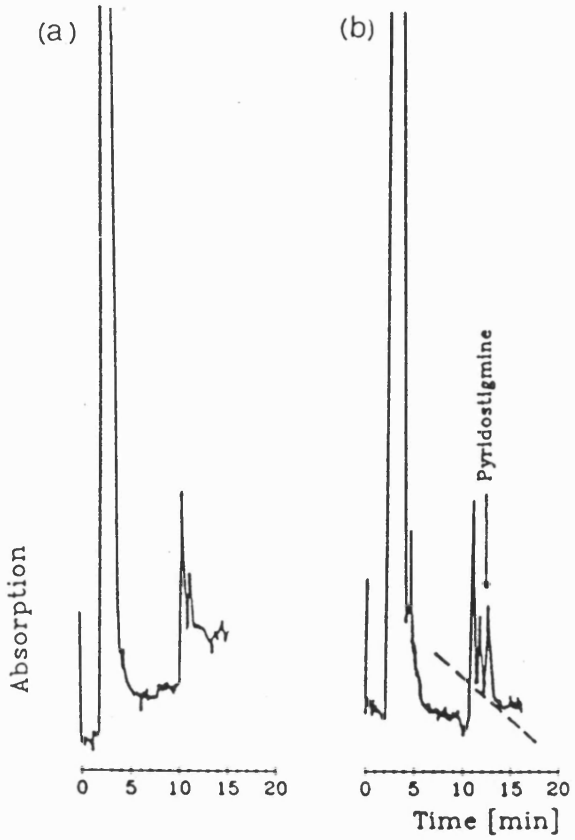


Fig 6.3 Chromatogram of (a) blank plasma (b) plasma sample 10 ng ml^{-1} 'tangent skimming' (Michaelis, 1990)

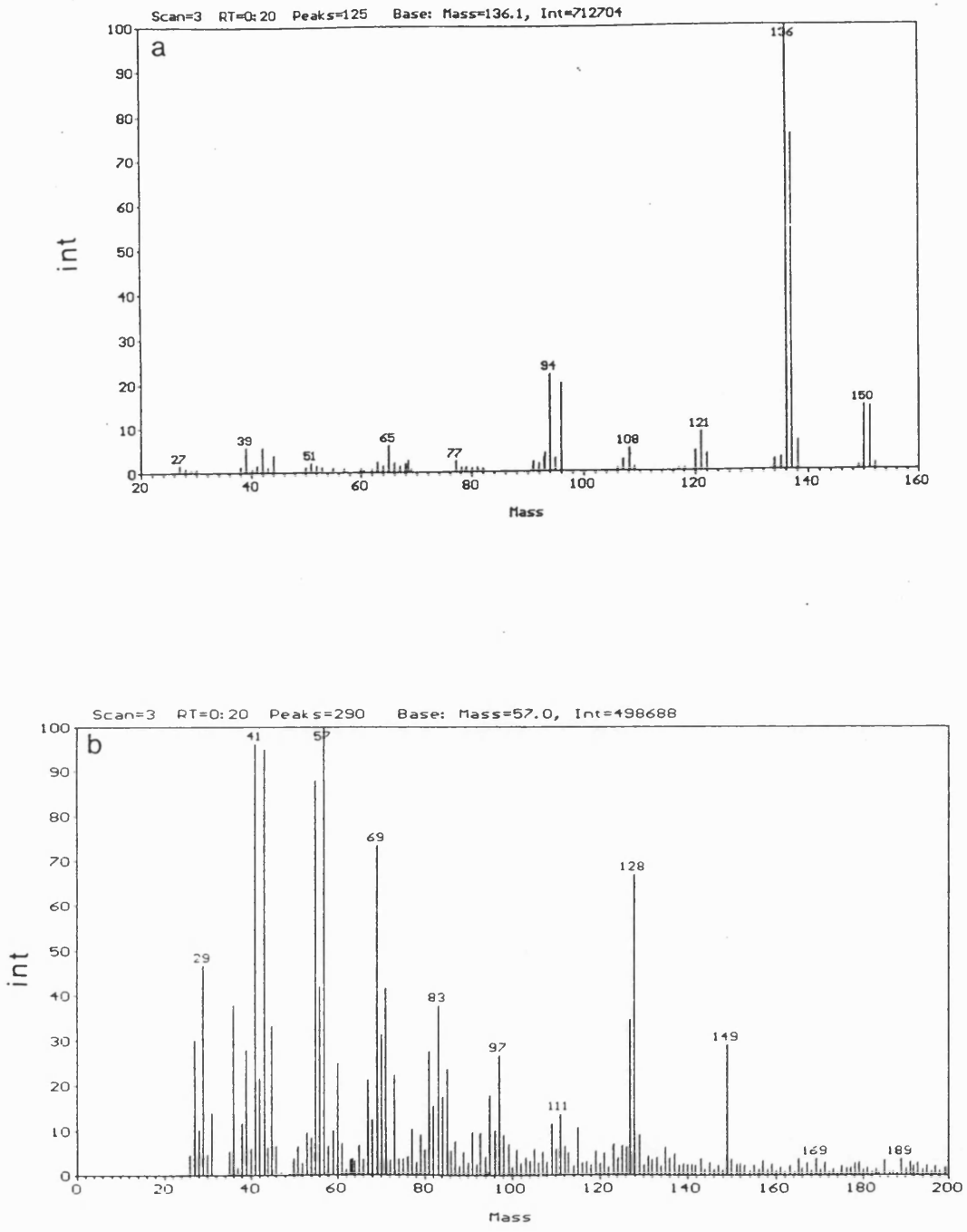


Fig 6.4 Mass spectra of the hydroxy metabolites of (a) neostigmine (b) pyridostigmine

6.7 Results and discussions

6.7.1 Chromatography on silica based columns

Initial attempts were made on SGE Cyano and SGE C_8 columns but both these columns showed considerable tailing. The addition of 0.02 - 0.002 % TEA to the mobile phase to reduce peak tailing caused the background signal to become too high to make any measurements.

A Hypersil BDS C_{18} (Shandon) column which is marketed as a base-deactivated column was tried and initially it gave good symmetrical peaks (TABLE 6.1) but proved unstable and started giving badly tailing peaks for quaternary ammonium compounds after 4 months (TABLE 6.2). The column was tested with the test mixture supplied by the manufacturer when it was received and 4 months later (TABLE 6.3).

TABLE 6.1
Column : BDS Hypersil 150 X 2.1 mm i.d.
Mobile phase: 8% ACN in 0.01M KH_2PO_4 pH 4.0 with 1.5 X 10^{-5} M DAS

	OHMN	Edrophonium	Neostigmine
κ	1.8	2.4	6.2
$w_{1/2}$	2.0	2.6	4.2
As_{10}	2.0	2.5	2.42
plates/metre	n.d	n.d	10854

TABLE 6.2 Same column 4 months later

	OHMN	Edrophonium	Neostigmine
κ	2.2	4.1	n.d
$w_{1/2}$	3.8	4.2	n.d
As_{10}	5.0	3.57	n.d

n.d not done.

TABLE 6.3 Column tested with manufacturers test mixture

Column: as TABLE 6.1

Mobile phase: 70% MeOH- water

Test mixture: supplied by the company

Date	Plates per metre	h
17 Oct 1990	51309	3.90
6 Feb 1991	22509	9.10

Fig 6.5 shows a chromatogram of some quaternary ammonium compounds on this column.

The hydrophilic hydroxy metabolite of pyridostigmine gave very poor sensitivities under post-column conditions i.e detection limit around 6.2 $\mu g\ ml^{-1}$. Since it is present in the urine in very small amounts work with this compound was not continued.

Ascah & Wilson (1990) had separated quaternary ammonium compounds using a Suplex pKb 100 column with a mobile phase consisting of acetonitrile-10mM Na_2HPO_4 pH 7.0 with 5mM sodium dodecylsulphate (30-70 v/v) at 210 nm. An attempt was made using a column which had previously been used for cocaine and its metabolites (Chapter 5).

With the mobile phase of acetonitrile-0.05M KH_2PO_4, pH 4.0 (15-85 v/v), all compounds eluted with the solvent front. To retain the analytes on the column the percentage of acetonitrile was reduced, moreover dodecyl- sulphate was replaced with DAS ($40mgL^{-1}$) as an ion-pairing agent. This was done in order to evaluate the use of post-column ion-pair extraction with fluorescence detection in a simplified 2 pump mode. Dodecyl sulphate would also have competed with DAS in the post-column extraction step, and reduce sensitivity. TABLE 6.4 shows the results of an optimization study carried out with this column and Fig 6.6 shows a chromatogram obtained on this column under the best possible conditions.

As can be seen from TABLE 6.4. 9%ACN with 80mg DAS L^{-1} gave the best column performance and acceptable resolution between edrophonium and OHMN+pyridostigmine and between edrophonium and neostigmine, however the peak symmetry was less than that obtained with 11% ACN. The Supelco pKb-100 was unable to separate OHMN and pyridostigmine. The use of 80mg DAS/L was considered uneconomical and therefore no further work was carried out with this column.

TABLE 6.4

Column: Supelco pKb 100 250 X 2.1 mm i.d

Mobile phase: 0.05M KH_2PO_4 pH 4.0 with various percentages of ACN and varying amount of DAS.

Flow rate 0.4 ml min^{-1}.

% ACN	DAS mg L^{-1}		Pyrido*	OHMN	Edrop*	Neo*
11	40	κ α Rs plates/ metre As_{10}	.0.23	0.38 1.65 0.18	0..69 1.81 0.5	1.61 2.33 1.33 7906 1.33
9%	40	κ α Rs plates/ metre As_{10}	0.53 n.r	0.61 1.15	1.07 1.75	2.30 2.14 7113 3.33
9%	80	κ α Rs plates/metre As_{10}	1.38 n.r	1.53 1.12 n.r	2.69 1.84 1.78	5.84 2.17 4.1 9950 2.81

n.r not resolved κ' determined by injecting separate solutions.
Pyrido= pyridostigmine; Edrop = edrophonium; Neo = neostigmine

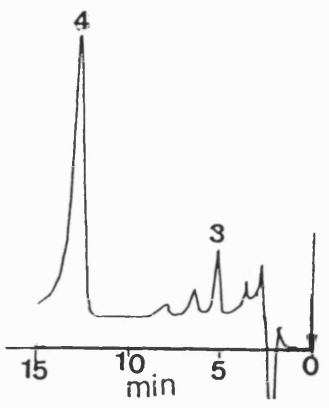


Fig 6.5 A sample chromatogram of (3) edrophonium (4) neostigmine on Hypersil BDS column

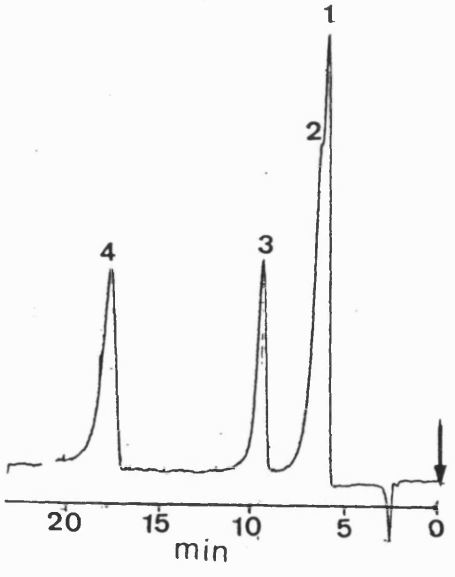


Fig 6.6 A Sample chromotaogram of quaternary ammonium compounds on Supelco pKb 100, under the best conditions (1) pyridostigmine (2) 3-hydroxy neostigmine, other codes same as Fig 6.5

6.7.2 Chromatography on PLRP-S column

More than 70% of separations in RP-HPLC are performed on alkyl-bonded silica based columns, however these columns have their limitations e.g lack of stability in aqueous mobile phases at high pH values, the presence of silanols, which may give rise to an unwanted phenomenon of tailing for acids and basic compounds, and the lack of compatibility with the biological activities of some proteins {poor protein recovery due to silanols} (Tanaka and Araki, 1989). One way around these problems is the use of polymeric columns which offer excellent chemical stability between pH 1-13 and complete absence of silanols.

Polymeric gels can be derivatized to give stationary phases for use in RP-HPLC, HIC (hydrophobic interaction chromatography), normal phase chromatography, ion-exchange chromatography and size exclusion chromatography without loss of chemical stability. PLRP-S is a macroporous polystyrene divinylbenzene (PSDVB) and is marketed by Polymer Laboratories (Shropshire U.K) for use in normal phase and RP-HPLC. In the reversed phase mode it is used for the chromatography of polar, ionic and ionisable solutes at pH 1-13.

One disadvantage of these columns is their limited stability to pressure, for example the maximum operating pressure for a 150 X 4.6 mm i.d column is 3000psi (Polymer Labs). Column efficiency is also limited and depends upon solute, solvent and temperature conditions, e.g acetonitrile and tetrahydrofuran cause swelling of the polymers and thereby improve column efficiencies, by reducing the volume surrounding each particle. On the other hand hydrophilic solvents e.g methanol cause shrinking of the polymer and show poor performance (Pedigo and Bowers, 1990 comments on 1986).

The retention mechanism on polymeric columns varies with the mobile phase used. Increasing the polarity of solvent changes the separation mode from adsorption to gel permeation and partition (Mori, 1978). A combination of adsorption and partition (Bowers and Pedigo 1986) and π–π interaction or charge transfer interactions (Tanaka and Araki, 1989) have been suggested. Whatever the dominant mechanism of retention during a given

separation, the retention on PSDVB columns will be different from alkyl based silica columns. This is because of the presence of aromatic rings and the lack of silanols on the polymer. Pedigro & Bowers, (1990) showed that with organic solvents like methanol, which are not adsorbed on the surface, the aromatic nature dominates, whereas for 'strong solvents' i.e THF and ACN which are adsorbed on the surface a similarity exists between polymeric and alkyl bonded silica phases. This may be the reason why aromatic compounds which have polar substituents give rise to 'tailing peaks' with alcoholic mobile phases. This tailing is independent of κ' values (Bowers and Pedigro 1986), and is less with 'deactivated' polymers in which C_{18} groups are bonded to aromatic rings (Benson and Woo 1984). Liedekerke (1989) has demonstrated the use of a polymeric column for the chromatography of quaternary ammonium compounds using aqueous acetonitrile with acceptable peak asymmetry (See Section 6.5), and so a polymeric column was examined.

With a mobile phase of acetonitrile - 0.05M KH_2PO_4, pH 3.0 (10:90v/v) only edrophonium and neostigmine were retained. To increase retention of other compounds. DAS was added to the mobile phase as an ion-pairing agent. Fig 6.7 shows the effect of an increase in DAS concentration on the retention of quaternary ammonium compounds. As expected, the retention increased with increasing concentration of the pairing ion and 9 X 10^{-5} M was preferred to give enough retention and an acceptable analysis time. Keeping the concentration of DAS constant, various organic modifiers were examined for their effect on column efficiency and selectivity.

TABLE 6.5 Percentage of various organic modifiers with 0.05M phosphate buffer pH 3.0.and a PLRP-S column. Flow rate 0.75 ml min^{-1}, wsf = with solvent front, nm= not measured.

Type of organic modifier	%	[DAS]10^{-5}M	κ' OHMN	κ' Edro	Plates/metre
ACN	10	5	1.0	1.8	13460
THF	9.44	5	wsf	wsf	nm
THF	5	5	0.33	0.36	nm
DCM in 10% ACN	0.5	5	0.53	1.06	7326

Acetonitrile (10%) was found to be the most suitable in terms of κ' values and column performance. Addition of 0.1 % DCM to ACN mobile phase did not improve the column efficiency. The analytes had no retention in presence of THF. The amount of DAS in the mobile phase was then kept constant and the percentage of acetonitrile in the mobile phase was varied. Fig 6.8 shows the results at 40° C, when the percentage of ACN was varied. Fig 6.9 shows a sample chromatogram of all the quaternary ammonium compounds at ACN-0.05M KH_2PO_4 pH 3.0; (11-89 v/v) containing 30 mg of DAS L^{-1} of mobile phase.

A new polymeric column 150mm X 2.00 mm i.d column was examined with the same mobile phase at flow rate of 0.4 ml min^{-1} but was found to have poor column efficiency. No further work was carried out with this column.

TABLE 6.6 : Column efficiency of 150 X 2.0 mm id column.

	κ'					
% ACN	DAS mg /L	Pyrid	OHMN	Edrop	Neo	plates/metre
11	30	0.7	0.9	1.45	3.2	14773
11	40	1.2	n.r	2.6	5.8	13177
9	40	2.1	n.r	4.4	10.5	12700

n.r not resolved * 20590 plates/meter for 4.6 mm i.d column

From the results on a 4.6 mm id column it is interesting to note that the order of elution i.e pyridostigmine, OHMN, edrophonium and neostigmine is similar to the one reported by Ruyter et al. (1986) on an alkyl silica based column using TMA^+Cl^- to neutralize silanols, which probably shows that under the experimental conditions used for this work there is no difference between a polymeric column and an alkyl bonded silica column. Edrophonium and OHMN both have hydroxy groups attached to the aromatic rings and are retained longer on the column than pyridostigmine which is a carbamate ester like neostigmine. It seems that the number of substituents attached to the N^+ have more influence on the retention of these compounds than the hydroxy groups (Fig 6.2). Probably with alcoholic mobile phases,

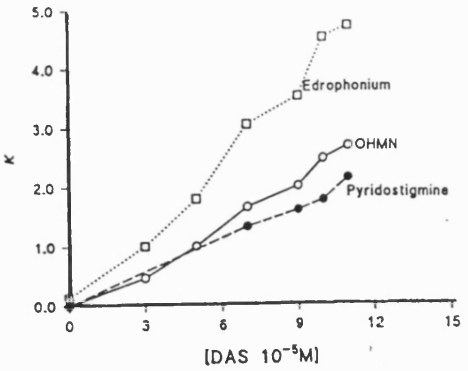


Fig 6.7 Effect of [DAS] on k'; PLRP-S 150 X 4.6 mm i.d. column

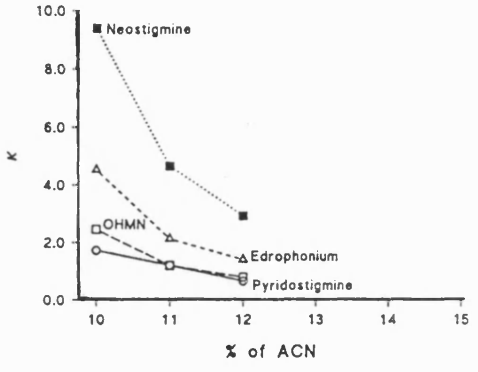


Fig 6.8 Effect of percentage of ACN on k'

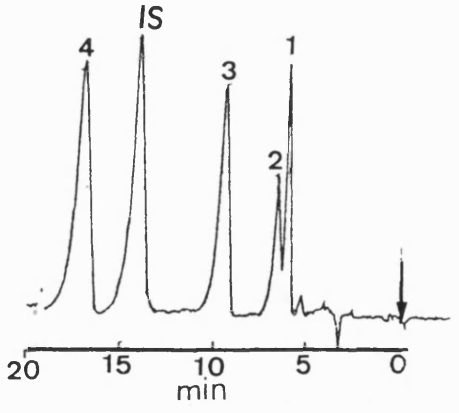


Fig 6.9 A sample chromatogram on PLRP-S column, (1) pyrid 0.34 µg ml^{-1}, (2) OHMN 0.72 µg ml^{-1}, (3) edrop 1.12 µg ml^{-1}, (IS) 1.12 µg ml^{-1}, (4) neo 0.72 µg ml^{-1} .

edrophonium and OHMN would have shorter retention than pyridostigmine and elute as badly tailing peaks on this column.

The resolution between pyridostigmine and OHMN was not very good under the best possible conditions. Because it is highly unlikely that both pyridostigmine and neostigmine would be present in the same samples, the chromatographic procedure was therefore optimised for two assays (a) pyridostigmine from plasma and (b) edrophonium, neostigmine and its metabolite from plasma.

6.8 Sample preparation

The sample preparation of positively charged substances can be carried out using either CBA (carboxylic acid) cartridges or SCX cartridges. For substances which are ionised at all pH values or have high pKa's e.g quaternary ammonium compounds, the use of SCX cartridges may become a problem, as to facilitate their elution from the cartridge (which if at all possible) would require the use of high ionic strength buffers and very high pH. On the other hand if CBA cartridges are used this 'elution' problem is avoided. CBA has a pKa of 4.8, above this pH it is negatively charged and can retain isolates, below pH 4.8 the charge is neutralised and elution is facilitated. The use of CBA cartridges was therefore preferred over other cartridges for sample preparation of quaternary ammonium compounds from plasma.

Initial attempts at sample preparation produced a huge peak with test cartridges which eluted with the solvent front under post-column conditions and was also observed at by UV at 254 nm. The presence of this peak was eventually traced down to the 'stainless steel' needles of the Vac Elut-24 manifold. For all the work in this chapter the stainless steel needles were replaced with 200µl Gilson pipette tips or 1 ml plastic tips.

6.8.1 Method

The cartridges were conditioned with MeOH and then with pH 8.0 phosphate buffer until the pH of the eluent was 8.0. The vacuum was stopped in order to prevent the cartridges from drying out.

Stock solutions of all the compounds were prepared in water and were stored at 4° C. Plasma 1.0 ml. was added to a 10.0 ml clean polypropylene centrifuge tube, spiked with known amounts of standard solutions, vortex mixed for 30 sec, diluted with 2.0 ml of phosphate buffer pH 8.0, and vortex mixed. It was then applied on the cartridge. Afterwards the cartridge was washed with 1.0 ml of pH 8.0 buffer, 1.0 ml of distiled water and 1.0 ml of MeOH. In the case of pyridostigmine, elution was carried out with 2.0 ml of 0.2% TFA in 70% MeOH. For other compounds elution was achieved with 0.2% TFA in methanol. The eluent was evaporated to dryness under a gentle stream of N_2 at 40° C, and made up in mobile phase.

Assay of pyridostigmine

For the assay of pyridostigmine the mobile phase used was ACN-0.05M phosphate buffer pH 3.0; (11: 89 v/v) containing 30 mg DAS/L at 30° C. The samples were made up in 1.0 ml of mobile phase and each injection was made in duplicate. Edrophonium was used as an internal standard. Peak height ratios were plotted against concentrations.

Fig 6.10 shows a sample chromatogram of spiked plasma, the lowest concentration of the calibration curve. The calibration curve was linear from 0.70 μg ml^{-1} to 0.035 μg ml^{-1}. The equations of calibration for aqueous standards and spiked plasma (±) standard deviation were:

Standard y=2.38 (±0.033) x + 0.046 (±0.012); r^2= 0.999 .(n=5)

Spiked sample y= 2.251(± 0.048) x +0.043 (± 0.025) ; r^2= 0.999 .(n=5)

The limit of detection for spiked plasma samples calculated according to Miller and Miller (1988) was 0.062 μg ml^{-1}. Much lower limits of detection (about 5 times lower) are possible if the sample was made in 200μl of mobile phase rather than 1.0 ml of mobile phase as was done in the present case.

Assay of edrophonium,neostigmine and metabolites

The mobile phase was similar to that used for the pyridostigmine assay except that the percentage of acetonitrile was increased to 11.5 and the temperature was raised from 30° C to 40° C. This was done to reduce the retention time of neostigmine. Initial attempts were made using gradient elution, so that a single analytical method could be used for pyridostigmine, edrophonium, neostigmine and its hydroxy metabolite. Unfortunately because of the presence of the fluorescent ion-pairing agent DAS in the mobile phase this approach was not suitable.

The samples were made up in 200 μl of mobile phase, and butyltripropyl ammonium bromide was used as an internal standard. The equations for calibration curves with (±) standard deviation for standard solution of all the analytes (range same as spiked samples) were:

OHMN $y=0.681 (\pm 0.037) x + 0.10(\pm 0.027); r^2=0.994$.

Edrophonium $y= 1.80 (\pm 0.11) x + 0.115 (\pm 0.053); r^2=0.994$.

Neostigmine $y= 4.13 (\pm 0.154) x + 0.034 (\pm 0.031); r^2=0.997$.

The sample chromatogram for spiked plasma sample is shown in Fig 6.11. The calibration curves were linear for spiked plasma from 1.40 - 0.084 μg ml^{-1} (OHMN); 0.81-0.081μg ml^{-1} (Edrophonium) and 0.4 - 0.03μg ml^{-1}(Neostigmine).

The equations for calibration and (±) standard deviation n=5 were:

OHMN:

$y = 0.613 (\pm 0.112) x + 0.09 (\pm 0.008)$; $r^2=0.999$ LOD 0.058 μg ml^{-1}.

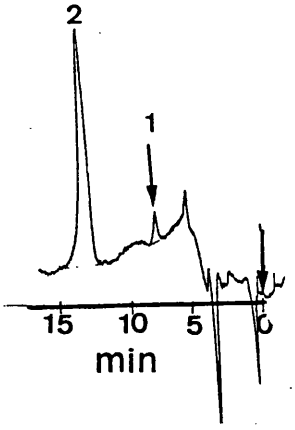


Fig 6.10 A sample chromatogram of spiked plasma from the calibration curve, conditions as specified in the text ; (1) pyridostigmine (2) edrophonium as internal standard.

a

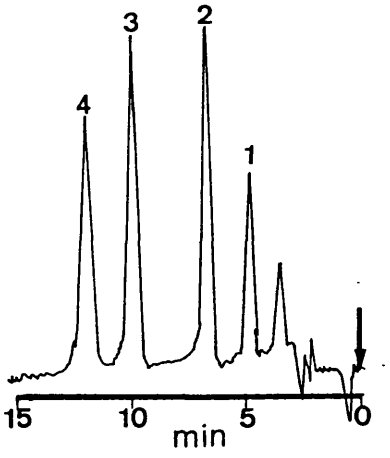


Fig 6.11 A sample chromatogram of spiked plasma from the calibration curve, (1) OHMN 0.70 µg ml^{-1}, (3) edrop 0.54µg ml^{-1}, (IS) 0.60 µg ml^{-1}, (4) neo 0.20 µg ml^{-1}.

Edrophonium:

$y= 1.69 (\pm 0.024) x + 0.143 (\pm 0.033)$; $r^2 = 0.994$ LOD 0.054 $\mu g\ ml^{-1}$.

Neostigmine:

$y = 4.039 (\pm 0.054) x - 0.01 (\pm 0.001)$; $r^2 = 0.999$ LOD 0.012 $\mu g\ ml^{-1}$.

The intercepts for OHMN and edrophonium are slightly positive, and the intercept for neostigmine is not different from zero. Repetition of the calibration resulted in similar results.

The extraction recoveries using 1.0 ml plasma sample as compared with the non extracted standards were:-OHMN 92.95%; edrophonium 93.71%; neostigmine 97.50 %;

The precision of the assay was calculated by injecting a sample six times. The results are shown as Pk ht ratio (±) standard deviation

OHMN 0.34 (±0.016) ; n=6; RSD 4.68%

Edrophonium 1.053 (±0.013); n=6; RSD 1.29%

Neostigmine 0.46 (±0.012); n=6; RSD 2.74%.

The Peak symmetries (As_{10}) were:- for OHMN 1.14, Edrophonium 1.55, BTA 1.18, and Neostigmine 1.09.

6.9 Conclusions

A solid phase extraction method has been developed for pyridostigmine, edrophonium, neostigmine and its hydroxy metabolites. The method developed in this study was found to be similar in general approach to the one developed by Matsunaga et al. (1987) for pyridostigmine. The recoveries for all the analytes were more than 90%. The sensitivity achieved for pyridostigmine is comparable to other published methods i.e., 10 ng ml^{-1} for Matsunaga et al. (1987). The limits of detection are higher for other quaternary

ammonium compounds i.e. 58 ng ml^{-1} for OHMN, 54 ng ml^{-1} for edrophonium and 12 ng ml^{-1} for neostigmine as compared to those obtained by Ruyter et al. (1980) i.e 5 ng ml^{-1} for neostigmine and edrophonium. The method is however more selective.

The polymeric column offers excellent properties for the chromatography of quaternary ammonium compounds using acetonitrile as an organic modifier. The As_{10} values for pyridostigmine and neostigmine are 1.0 and for edrophonium 1.54 and OHMN greater than 1.14 which are similar to the values obtained by Liedekerke (1989).

Post-column ion-pair extraction detection offers selectivity and less interference from sample components which are normally seen at low U.V wavelength i.e 214 nm. The sensitivity of the method can however be improved by using a modern HPLC fluorescence detector, which has not been available in this work.

7 EVALUATION OF SOME FLUORESCENT DYES AS POTENTIAL POST-COLUMN ION-PAIRING AGENTS

7.1 Introduction

The determination of drugs by fluorometric methods offers advantages of selectivity and sensitivity over UV methods especially for the determination of drugs at nanogram level in biological samples, either for therapeutic drug monitoring or forensic purposes. Fluorescence detection is very selective as very few compounds fluoresce naturally. For analytes which are non-fluorescent, this problem can be overcome by selective derivatisation of the compounds of interest, as discussed in the introduction (Section 1) with a reagent which can provide fluorescent properties to the compound. A common example of this is the derivatisation of aminoacids with OPA (orthophthalaldehyde) and mercaptoethanol, which can produce detection in ng-fg range. However as discussed in Section 1.3 of this thesis, some ionisable compounds cannot be derivatised in the usual way eg tertiary and quaternary ammonium compounds and some acids. One way forward for such substances is to ion-pair them with a suitable fluorescent ion that also has some hydrophobicity. The ion-pairs formed are more lipophilic and so are extracted into a suitable organic solvent and the fluorescence measured.

7.1.1 Principles of ion-pair extraction

A great deal of work on ion-pair extraction has been done by Schill and co-workers (1977) that is beyond the scope of this thesis to discuss. However some fundamental relationships which are relevant to this section are included here.

The extraction of a positively charged cation Q^+ such as an amine or a quaternary ammonium compound, with a fluorescent anion X^- from an aqueous solution into an organic liquid as an ion-pair Q X can be expressed by the formula:

$$Q^+_{aq} + X^-_{aq} \rightleftharpoons Q X_{org} \qquad \text{Eq 7.1}$$

where aq and org refer to aqueous and organic phases respectively

According to Schill (1978) a quantitative expression for the distribution of ion-pair Q X is given by the extraction constant E_{QX} which may be defined as:

$$E_{QX} = \frac{[QX]_{org}}{[Q^+]_{aq}[X^-]_{aq}} \qquad \text{Eq 7.2}$$

E_{QX} is a stoichiometric constant and its value will change with the nature of the phases. This equation assumes a 1+1 ion-pair formation.

Assuming that no side reactions occur i.e the extraction of Q^+ and X^- into the organic phase occurs only as Q X , then the distribution of Q between the two phases i.e., the aqueous phase and the organic phase can be given by the expression of the distribution ratio D_Q:

$$D_Q = \frac{[QX_{org}]}{[Q^+]} = E_{QX}[X^-] \qquad \text{Eq 7.3}$$

The above equation indicates that D_Q will change with the change in the concentration of the counter-ion $[X^-]$. The value of the constant E_{QX} can be changed not only by changing the nature of the organic phase but also by changing the nature and concentration of the counter-ion. A quantitative extraction (>99%) is obtained when the distribution of D_Q is > 100 and equal phase volumes are used.

Factors affecting ion-pair extraction (Tomlinson,1983)

1 Nature of extracting phase: Polarity
presence of adduct forming agents
volume

2. Nature of aqueous phase: pH
salt concentration
volume

3. Nature of the ion pairing agent: physical properties
optical properties
concentration in aqueous phase.

4. Temperature

There are many choices available regarding factors 1 and 2, but very few counter-ions are available. According to the above discussion, a considerable increase in detector response can be obtained in post-column ion-pair extraction systems by proper choice of counter-ion.

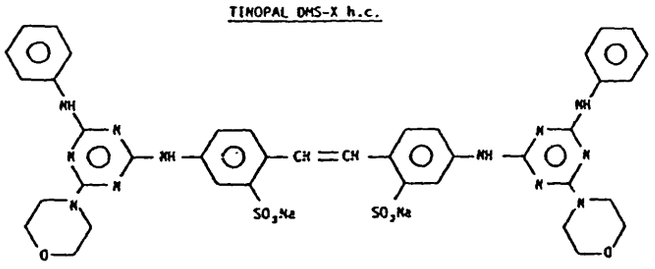
There may be a large number of fluorescent dyes which can be used as potential post-column ion-pairing agents but very few have been evaluated for actual use as post-column ion-pairing agents. Some of the fluorescent dyes which have been used are:

Reagent	Application	References
Acridinium chloride	anionic surfactants	Smedes et al. (1982)
DAS	tertiary and quaternary ammonium compounds	Westerlund & Borg (1973)
Tinopal G.S	erythromycin	Tusji (1978)
Sodium α-phenyl cinnamonitrile sulphonate	physostigmine and quaternary ammonium compounds	Quinn (1989)

It is interesting to note that in a review article on fluorescence detection in HPLC by Lingeman et al (1985), out of 672 references, DAS has been used as post-column ion-pairing agent in only 8 cases and no other post-column ion-pairing agent has been mentioned. This can mean that either there are very few applications where post-column ion-pair extraction system can be used to increase sensitivity or that very few fluorescent counter-ions are available for use. Considering that most drugs are ionisable compounds the second possibility seems more likely.

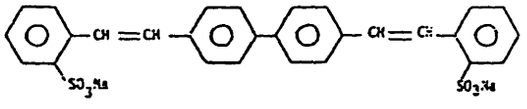
The aim of the work in this study was to evaluate some fluorescent dyes for use as post-column ion-pairing agents and compare them with DAS, which has been used in earlier chapters of this thesis. The dyes that were examined were: Tinopal GS, Tinopal DMS

TINOPAL DMS-X h.c.



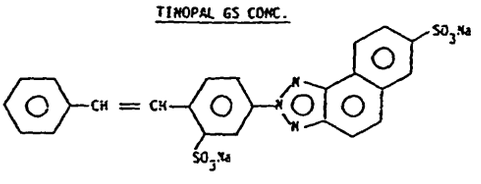
A DIAMINOSTILBENE CYANURIC CHLORIDE DERIVATIVE

TINOPAL CBS-X



DISTYRYL BIPHENYL DERIVATIVE

TINOPAL GS CONC.



DISODIUM - 4 - (2H - 6 - SULPHONAPHTHO [1,2 - d] TRIAZOL - 2 - YL)
STILBENE - 2 - SULPHONATE

RBS-200

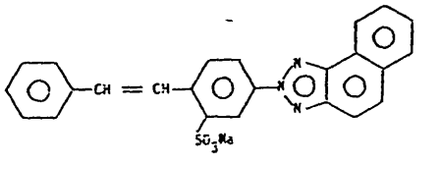


Fig 7.1 Structures of fluorescent dyes examined.

conc, Tinopal CBS-X , and Tinopal RBS 200. The structures are given in Fig 7.1. These dyes are used as fluorescent brighteners in washing powders, and soap. Only Tinopal GS has been used as a fluorometric reagent. For a fluorescent dye to be used as a potential post-column ion-pairing agent certain properties need to be examined.

1. Physical properties and structure.
2. Optical properties.
3. Its ability to form extractable ion-pairs i.e its extraction constants.

7.2 Physical properties

1. Solubility:

The solubility of an anion in water is very important, and will influence the range of concentration in which it can be used.

2. Hydrophobicity:

The more hydrophobic the pairing ion is, the greater will be the extraction constant, and the greater the transfer of the formed ion-pair into the extracting organic phase. Alternatively a very large and hydrophobic molecule cannot be used as it will form ion-pairs with other sample components e.g Na^+ etc. and give very large blanks thus compromising the sensitivity of the method. Hydrophobicity will affect the aqueous solubility of the ion-pairing agent.

3. Structure of ion-pairing agent:

A great deal of work has been done on the structures of ion-pairing agents for the extraction of basic compounds by Modin & Schill, (1967) and Westerlund & Borg (1970), which can be summarised as:

1) Sulphonates generally yield ion-pairs with higher extraction constants than carboxylates and phenols, because they are very strong acids, and they be used over a wide pH range.

2) The ion-pairing agent must not have hydrophilic groups other than sulphonates. They also must have a large number of alkyl or aryl carbon atoms.

3) A conjugated π electron system is necessary to give excitation and emission in a wavelength region used in common analytical instruments.

4) Halogen, nitro-, and carboxyl substituted compounds are not used as they tend to decrease fluorescence.

7.3 Optical properties

The principles and theoretical description of fluorescence is discussed by many textbooks e.g Rabek, (1982) and reviews (Lingeman et al, 1985). However, to get an understanding of optical properties a brief description of fluorescence and various terminologies involved is given.

Fluorescence is the emission of light which occurs when an electronically excited molecule returns to its ground state. Not all molecules return to their ground state by the emission of light e.g aliphatic molecules which have a high degree of vibrational freedom return to their ground state by vibrational relaxation and no fluorescence is observed. On the other hand for aromatic and highly conjugated molecules which have a low degree of vibrational freedom, the vibrational relaxation path is not effective and the return to ground state is accomplished by emission of radiation. However not all molecules which are excited emit light as they try to reach their ground state, otherwise the intensity of fluorescent (I_f) would be the same as the intensity of absorbed light (I_a). This is because of several processes which compete with each other so that the molecule returns to its ground state or lowest excited state, the intensity of fluorescence (I_f) is thus a fraction (φ) of the absorbed light.

$$I_f = \varphi\ I_a. \qquad \text{Eq 7.4}$$

φ is called the quantum yield of fluorescence and its value is far from unity. For quinine bisulfate, a good fluorescent molecule, its value is 0.55. For fluorescence to be observed by instruments the value of φ should be more than 0.01. The fluorescent properties of a compound are studied by examining its excitation and emission spectra.

Combining Eq 7.4 with Beers law the following equation is obtained:

$$I_f = \varphi \cdot I_o (1\text{-}10^{-\varepsilon cl}). \qquad \text{Eq 7.5}$$

The above equation means that the intensity of fluorescence is by definition equal to the intensity of absorbed light measured in quanta, multiplied by quantum efficiency of fluorescence, where I_0 is the intensity of incident light (quanta/sec) for which the compound has a molar extinction coefficient ε (Parker & Rees, 1960).

The above equation indicates that I_f is not linear with analyte concentration, but in dilute solutions where the value of εcl is less than 0.02, the above equation can be simplified to:

$$I_f = 2.3\, I_o\, \varphi .\, \varepsilon cl \qquad \text{Eq 7.6}$$

Eq 7.6 shows that for a given solution the fluorescence intensity is proportional to $I_o.\varepsilon.\varphi$. For many substances the quantum yield of fluorescence is independent of the excitation frequency. Thus if the intensity of incident light in quanta per second is kept constant and the frequency is varied, the fluorescent intensity is proportional to the molar absorptivity ε of the compound (Parker and Rees 1960).

Unlike absorption studies where ε provides much information about the absorption behaviour of a compound, the quantum yield of fluorescence φ, in itself, is not a good parameter to quantify the fluorescent sensitivity of the compound as it provides no information on the number of photons absorbed. The molar absorptivity i.e total number of photons absorbed and the band width at half height of the emission spectra (H) are also important to quantify the fluorescent sensitivity of the compound.

Parker and Rees (1960) have described a way for measuring fluorescence sensitivity and called it the fluorescence sensitivity index (FSI)

$$FSI = \frac{\varphi\ \varepsilon}{H} \qquad \text{Eq 7.7}$$

The half band width (H) is calculated from the emission spectrum. The units of H are in wave number ($\nu' = 1000/\lambda$) . The quantum yield of fluorescence can be measured either by absolute methods or by relative means.

Determination of absolute quantum yields is difficult and rarely done because it requires a lot of expensive, complicated instruments and involves a lot of complex mathematical formulae (Rabek,1982). The relative method is much simpler as it does not involve complicated equipment. It is also more accurate than the absolute method. In this method the substance to be examined is compared with a standard substance whose quantum yield is known. To calculate the quantum yield of an unknown substance φ_x the following equation is used.

$$\varphi_x = \frac{I_x \varepsilon_s c_s}{I_s \varepsilon_x c_x} \times \varphi_s \qquad \text{Eq 7.8}$$

where x and s are unknown and standard respectively, and I is the fluorescence intensity.

The Table gives a list of some of the substances that can be used as standards for quantum yield measurements. Data taken from Parker and Rees (1960)

Compound	Solvent	Quantum yield
Fluorescein	Aqueous carbonate-bicarbonate buffer pH 9.6	0.85
Rhodamine B	Ethanol	0.69
Quinine bisulfate	0.05 M H_2SO_4	0.55
Eosin	0.1 N NaOH	0.23
Anthracene	Ethanol	0.28

If the concentration of solutions is regulated in such a way that $\varepsilon_1 c_1 = \varepsilon_2 c_2$. and solutions are very dilute so that $\varepsilon c < 0.01$ (From Calvert and Pitts, the subscripts 1 and 2 refer to standard and sample respectively). The above equation then reduces to:

$$\varphi_x = \frac{I_x}{I_s} X \varphi_s \qquad \text{Eq 7.9}$$

where I_x is the area under the true fluorescence emission curve of the compound under study and I_s is the area under the true curve for the standard. (ibid)

$$\varphi_x = \frac{\text{Area under the curve of the unknown}}{\text{Area under the curve of standard}} X \varphi_s$$

When different solvents are used for standard and sample, the fluorescence is multiplied by the square of the refractive indices (n^2) for the solvents in calculating the quantum yields (Hermans& Levinson, 1951).

$$\varphi_x = \frac{Ix\,(n^2)_x}{I_s(n^2)_r} X \varphi_s \qquad \text{Eq 7.10}$$

Fluorescence spectra which are obtained on ordinary spectrofluorometers are usually 'apparent emission spectra' or 'uncorrected spectra' and are generally plots of relative fluorescent intensity 1-100 in arbitrary units vs wavelength. However both the emission of xenon arc as well as detector sensitivity are dependent on wavelength thus distorting the recorded spectra. The 'apparent emission spectrum' is therefore not a direct measurement of the number of quanta emitted at each frequency in a fluorescence band, and a correction to the observed phototube response must be made to obtain values proportional to the number of quanta per sec. This is a major problem in the determination of relative quantum yields.

7.4 Methods of correction of spectra

The manual correction of spectra is a difficult and time-consuming procedure, and even after corrections are made the results are only accurate to ± 10% (Lackowicz, 1983).

Present day modern research grade spectrofluorometers can be fitted with devices which can measure corrected emission spectra but these devices are costly. For older instruments a number of manual correction methods are available:

a) Standard lamp method

b) Quantum counter and light scatterer.

c) Standardised solution method.

A brief description of the methods is given below (Lackowicz, 1983)

a. Standard lamp method

In this method, correction factors for emission spectra are obtained from the measurement of a standardised lamp projected through the sample compartment, dispersed by a grating and measured with a phototube. (White and Argauer, 1970) Standard lamps of known colour temperature and the spectral output data [L (λ)] are available from the National Bureau of Standards. The detection system is calibrated as follows:

1. The intensity of standard lamp vs wavelength I (λ) is measured using the detector system of the spectrofluorometer.

2. The sensitivity of the detection system S (λ) is calculated using

$$S(\lambda) = I(\lambda) / L(\lambda)$$

3. The corrected spectra are then obtained by dividing the measured spectra by the sensitivity factors.

Apart from being expensive this approach is complicated as the operation of the standard lamp requires precise control of the colour temperature and the spectral output of the lamp varies with time and usage.

b. Quantum counter and scatterer

In this method the spectral output of the lamp is determined and then this lamp is used as a calibrated light source. The relative photon output L (λ) is obtained by placing a quantum counter e.g (Rhodamine B in ethylene glycol 3 gm/L) in the sample compartment. Once this intensity distribution is known, the xenon output is directed onto the detector using a magnesium oxide scatterer. The procedure is as follows:

1. The excitation wavelength is scanned with the quantum counter in the sample holder. The output yields the lamp output L (λ) .

2 . The scatterer is placed in the sample compartment and the excitation and emission monochromators are scanned in unison. This procedure yields the product S (λ) L (λ) where S (λ) is the sensitivity of the detection system.

According to Lackowicz, (1983) although the procedure seems very simple it is quite complicated to operate in practice, and it is difficult to obtain a reliable scatterer.

c. Standardised solution method

Compared to the methods mentioned above this method is relatively simple and reliable and was followed in this thesis. Fluorescent spectra of many 'standard' substances have been published which were recorded on a standard instrument in the range of 300- 800 nm. and the data is available in graphical and numerical form as Q/Q_{max} (see appendix Table I). To obtain correction factors for a particular instrument one records the emission spectra of the standards on that particular instrument. The emission curves as obtained on the chart recorder are then divided by Q/Q_{max} values to give relative fluorescent emission correction values in reciprocal quantum units. Subsequent traces of compounds fluorescing in the range of these standards can be corrected by dividing the traced values by the correction factors obtained earlier. The method is explained more fully in results and discussion by means of an example.

7.5 Extraction constants

An ion-pairing agent should have good optical properties and be able to form extractable ion pairs.

A comparison of the extractability of ion-pairing agents can be made by measuring their extraction constants E_{QX} and their distribution constants with a model cation e.g. TBA .

$$E_{QX} = \frac{QX_{org}}{Q^+_{aq} \cdot X^-_{aq}} \qquad \text{Eq 7.2}$$

$$D_Q = E_{QX} [X^-] \qquad \text{Eq 7.3}$$

An increase in the reagent concentration leads to an increase in the extraction, until it reaches a point where no calculated change in extraction efficiency is obtained. This is the optimum concentration for the ion-pair extraction. This ignores other factors such as high blanks or background signal, where a balance needs to be maintained between an improvement in the extraction and the background signal. In determining extraction constants it is necessary to determine that E_{QX} is constant over the concentration range of Q^+. The higher the value of E_{QX} or D_Q the greater will be the distribution of the ion-pair into the organic liquid.

The principles and the techniques for the determination of extraction constants and the mathematical relationships involved have been developed by Schill and co-workers (1978). Although his work mainly used batch extraction studies, the same principles can be applied to flow-injection-analysis or FIA. as shown by Johansson et al (1980).

7.5.1 Methods for determination of extraction constants

In batch extraction studies the aqueous ion-pairing agent at a suitable pH, is spiked with a known concentration of TBA (tetrabutyl ammonium hydroxide) and is shaken with an immiscible organic liquid (equal phase volumes) for 20 minutes using a mechanical shaker, or vortex mixed for 1 minute (Quinn 1989). After phase separation and centrifugation the layers are separated and the concentration of X^- now as the ion-pair

C'_{ox} is measured in the organic phase. Assuming a 1+1 ion-pair complex is formed, this can be written as:

$$E_{QX} = \frac{C'_{QX\ org}}{(C'_{Q^+}.\ C'_{X^-})_{aq}} \qquad \text{Eq 7.11}$$

$$C'_{QX\ org} = C_{Q\ org} = C_{X\ org}$$

The concentration of the anion C'_{X^-} and the concentration of the cation C'_{Q^+} remaining in the aqueous phase is calculated from:

$$C'_{X^-} = C^{\circ}_{X^-} - C'_{QX} \qquad \text{Eq 7.13}$$

and

$$C'_{Q^+} = C^{\circ}_{Q^+} - C'_{QX} \qquad \text{Eq 7.14}$$

where C° is the initial concentration of Q^+ and X^- respectively. The equation assumes that equal phase ratios are being used, and that X^- and Q^+ are extracted into the organic phase only as an ion-pair. The extraction of X^- in forms other than the ion-pair with Q^+ is compensated for by blanks. The concentration of QX in the organic phase is generally read from the calibration plot.

The problems with batch extraction studies are long shaking times, especially with the use of mechanical shakers, preparation of calibration plots for the measurement of ion-pair in the organic phase and the presence of water droplets in the organic solvent after centrifugation which may give erroneous results.

Johansson et al. (1980) using picrate as an ion-pairing agent with U.V detection described a method for the determination of extraction constants using flow injection analysis. In this method, the aqueous phase containing the ion-pairing agent and the organic phase are pumped continuously as small segments through an extraction coil where ion-pair extraction takes place. The test compound is generally injected into the aqueous phase containing the ion-pairing agent before it mixes with the organic liquid.

After leaving the extraction coil and phase separation the organic phase is pumped through the flow cell of the detector and the extraction constants are calculated by slope analysis from the experimental data i.e absorbance and counter-ion concentration. The authors have given the following equation (7.15) for the calculation of extraction constants:

$$1/A = (rD_{pre}D_{post}/\varepsilon l.C^{\circ}_{HB}) + (D_{pre}D_{post}/\varepsilon l.C^{\circ}_{HB}.K_{ex(HBP)})[P]^{-1} \qquad \text{Eq 7.15}$$

Where A= absorbance at peak maximum, r= phase volume ratio, C°_{HB}= initial concentration of organic ammonium ion (Q^+) or the injected sample. $D_{pre}D_{post}$ = dispersion of the sample before and after the extraction coil , $K_{ex(HBP)}$ is the extraction constant of HBP and P is the concentration of the dye (X^-).

A plot of 1/A vs 1/P will give a straight line with the intercept.

$I = rD_{pre}D_{post}/\varepsilon l.C^{\circ}_{HB}$ and slope $S = D_{pre}D_{post}/\varepsilon l\, C^{\circ}_{HB}.K_{ex\,(HBP)}$

and $I/S = r.K_{ex\ HBP}$

The plot of 1/A vs 1/P will not give a straight line, if side reactions occur e.g dissociation or dimerization of ion-pair in the organic phase, since $K_{ex\ (HBP)}$ changes with A i.e the total concentration of the ion-pair in the organic phase. According to the authors the results of extraction constants obtained by this method usually agreed ±0.1 log units with those obtained by batch extraction studies. The method can measure extraction constants in the range $1.1 < A_{max}/A_{min} < 9.0$.

7.6 Materials and Methods

Tinopal GS, Tinopal CBS-X, Tinopal DMS conc, and RBS-200 are referred collectively as test dyes in this section. For quantum yield measurements quinine bisulphate, Tinopal CBS-X, Tinopal GS, were prepared in 0.05 M H_2SO_4. The solutions were prepared so that $\varepsilon 1c1 = \varepsilon 2c2$ ($\varepsilon cl < 0.01$) and were excited at 335 nm (Westerlund and Borg 1973). The

emission spectra were corrected using the standard solution method as described (see results and discussion) earlier. The areas under the curve were determined by cutting and weighing.

Tetrabutyl ammonium hydroxide 0.1M was prepared by dissolving a known amount of tetrabutyl ammonium bromide and twice the amount of silver iodide in absolute methanol and stirring the solution for 15 minutes. After filtration the solution was collected in an amber coloured flask and protected from the light.

For the determination of quantum yields of TBA ion pairs, stock solutions of DAS, and other ion-pairing agents were prepared in water. Dilution of DAS and other ion-pairing agents were made in phosphate buffer (0.1M NaH_2PO_4). In a 15ml silanized glass tube, 5 ml of the ion-pairing agent, 50µl of 0.1M TBA and 5ml of dichloromethane were added. The mixture was vortex mixed for 30 seconds and left for 15minutes. It was then centrifuged for 10 minutes at 2000 rpm, the organic phase was removed with a syringe previously rinsed with ethanol and collected in a volumetric flask containing 10^{-3} M TBA and made up to volume with ethanol. The fluorescence spectrum of the ethanolic solution was measured.

The determination of extraction constants was performed at ambient temperatures using the method of Johansson et al (1980) in Mode 2 i.e the cation is injected into the aqueous stream of an anion before mixing with an immiscible organic liquid. The flow injection set up is described in Chapter 2 of this thesis. The absorbance measurements were made at the excitation wavelengths of the dyes and using a Spectra Physics Integrator Model SP 4270.

The determination of extraction constants by batch extraction studies was performed as described earlier in the methods for determination of extraction constants (7.4.1) i.e the samples were vortex mixed for 1 minute, centrifuged and the layers separated. The concentration of the dye in the organic phase was measured at its respective excitation

and emission wavelength using a Perkin Elmer 204 S fluorescence detector which has been used in the earlier chapters of this thesis.

7.7 Results and Discussions

7.7.1 Physico- chemical properties.

The dyes under test (Fig 7.1) were disulphonic acids apart from RBS 200 which is a mono-sulphonic acid. They are large molecules (RMM > 450), relatively hydrophobic without additional functional groups, and with a large number of aryl carbon atoms. They meet most of the criteria as described in Section 7.2.

At ambient temperature, all the dyes were soluble in water at a concentration of 1 X 10^{-4} M (the concentration at which DAS is generally used), apart from 'Tinopal DMS conc' which was completely insoluble in boiling water at this concentration or lower. The solutions of these dyes were prepared according to their relative purity apart from RBS-200 which was used at 50 mg/L in the absence of information regarding its strength or purity. Tinopal DMS conc was omitted from the study, because of its insolubility in water.

As mentioned earlier (Section 7.1) Tinopal GS has been used for the fluorometric determination of erythromycin, however its optical properties and extraction constants were not determined.

7.7.2 Optical properties

For the determination of optical properties it is important that substances should be as pure as possible because very dilute solutions (A < 0.01) are required for fluorescence measurements and the presence of impurities can give erratic results. Unfortunately these test dyes were not available in pure form.

The composition of these dyes was as follows:

Tinopal GS: 69 % active, 20% sodium chloride and 11% water, CBS-X was 90% active. Compensations for their purity were made by adjusting their concentrations according to their active strengths. Tinopal GS and RBS-200 are similar in structure (apart from the difference in the number of sulphonic acid groups, GS has got two sulphonic acid groups whereas RBS 200 has one.) and would have similar optical properties.

Correction of Spectra

For the correction of spectra the fluorescence emission of the three standards 3-aminopyridine (350-400nm), quinine sulphate (400-550nm) and 3-aminophthalimide (550-630nm) were recorded on the Shimadzu RF540. The emission readings obtained for the standards at each wavelength were then divided by Q/Q_{max} values from TABLE A 1 in Appendix II to get correction factors which are given in TABLE 7.1-7.3 below.

The 'apparent emission spectra' of DAS, CBS-X and Tinopal GS aqueous solutions shown in Fig 7.2 (a), (b) and (c) were obtained on the above instrument and the emission values obtained were then divided by the correction factors to give the corrected emission spectra as shown in Fig 7.3 (a), (b) and (c)

Fig 7.4 & 7.5 (a), (b) and (c) show the uncorrected and corrected emission spectra of TBA ion -pair with various reagents.

TABLE 7.4 gives the method of correction of spectra for CBS-X aqueous solution. For the correction of other spectra see Appendix II.

TABLE 7.1

2-Aminopyridine 1×10^{-5}M in 0.1 N H_2SO_4

Excitation at 228 nm

λ nm	Q/Qmax	Shimadzu	Correction factors
320	2.5	2.0	0.8
330	9.5	13.1	1.38
340	33.0	31.2	0.945
350	66.5	47.9	0.725
360	94.0	55.0	0.585
368	100	54.70	0.547
370	99.5	53.7	0.549
380	91.8	46.0	0.501
390	76.0	35.5	0.467
400	53.5	25.4	0.477

TABLE 7.2

Quinine Sulphate $1\mu g ml^{-1}$ in 0.05 M H_2SO_4

λ	Q/Qmax	Shimadzu	Correction factors
400	11.8	7.5	0.6356
410	26.7	14.2	0.5318
420	47.8	22	0.4620
430	68.3	28.2	0.4129
440	85.5	31.6	0.3696
450	95.4	31.9	0.3344
460	100	29.4	0.294
470	98.3	25.3	0.2574
480	90.0	20.5	0.2278
490	78.8	16.0	0.2030
500	70.0	12.1	0.1728
510	58.5	8.9	0.1521
520	49.0	6.2	0.1265
530	42.0	4.0	0.0952
540	33.2	2.3	0.693
550	29.1	1.4	0.0481

TABLE 7.3. 3-Aminophthalimide Ex 390 1μg ml^{-1} in 0.05M H_2S0_4

λ nm	Q/Qmax	Shimadzu	Correction Factors
550	71.6	12.53	0.175
560	60.8	7.76	0.127
570	56.5	4.48	0.08
580	40.0	3.0	.075
590	34.0	2.09	.061
600	27.0	1.20	.04
610	16.6	0.6	.036
620	13.5	0.6	.022
630	10.4	0.6	0.019

TABLE 7.4 Calculation of corrected emission spectra for CBS-X

λ nm	Shimadzu	C.F*	Corrected readings
400	35.82	0.6356	56.35
410	48.95	0.532	92.05
420	59.01	0.462	127.93
430	64.47	0.413	156.2
440	60.89	0.37	164.8
450	54.32	0.3344	162.5
460	46.56	0.294	158.4
470	37.61	0.2574	146.1
480	28.66	0.228	125.8
490	21.79	0.203	107.3
500	15.82	0.1728	91.6
510	11.94	0.152	78.5
520	8.35	0.126	66.1
530	5.37	0.0952	56.4
540	3.58	0.0693	51.7
550	2.08	0.048	43.4

CF* are the correction factors obtained from TABLE 7.1-7.3

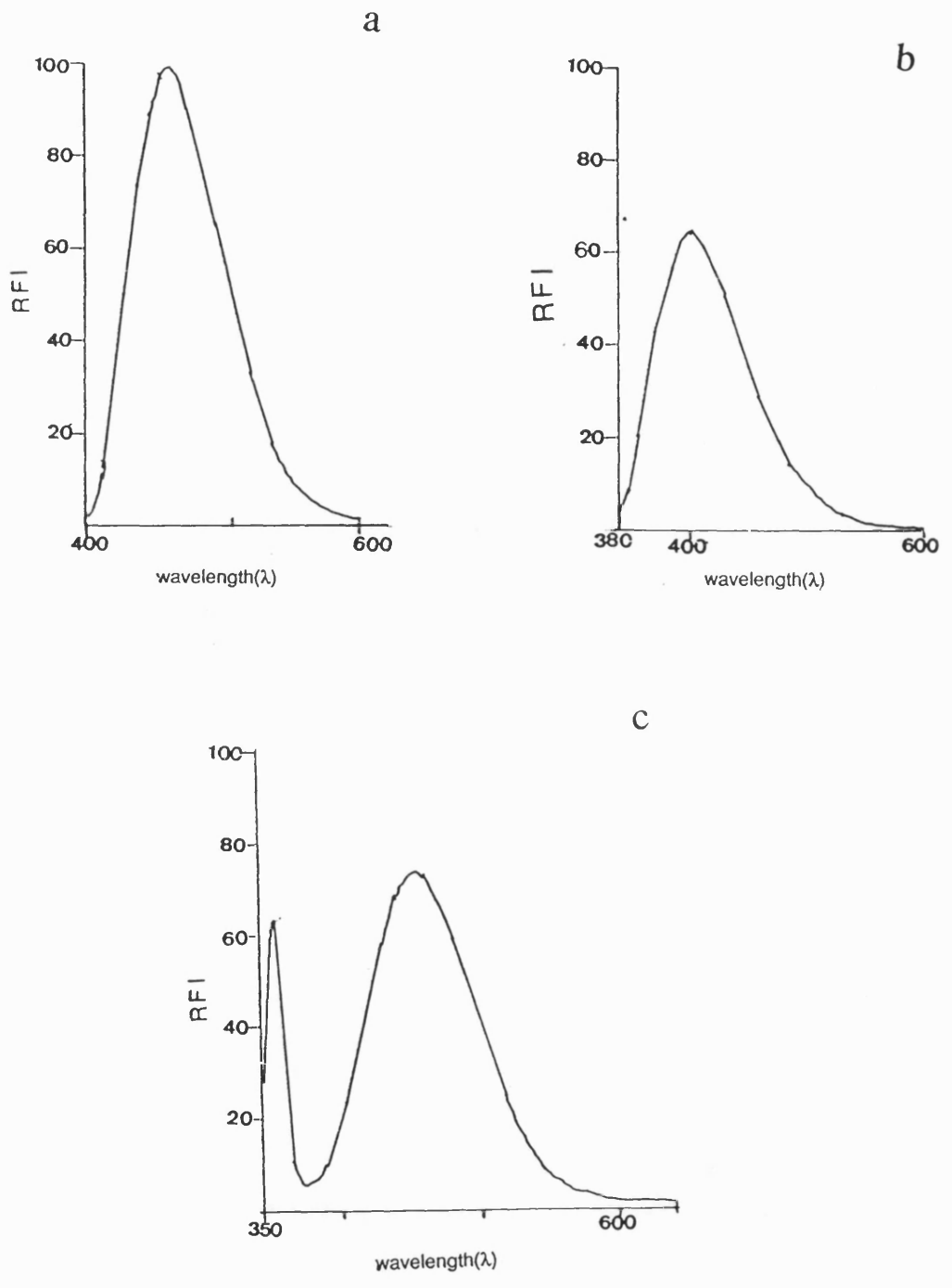


Fig 7.2 Apparent emission spectra (a) DAS, (b) Tinopal CBS-X , (c) Tinopal GS. RFI= relative fluorescent intensity.

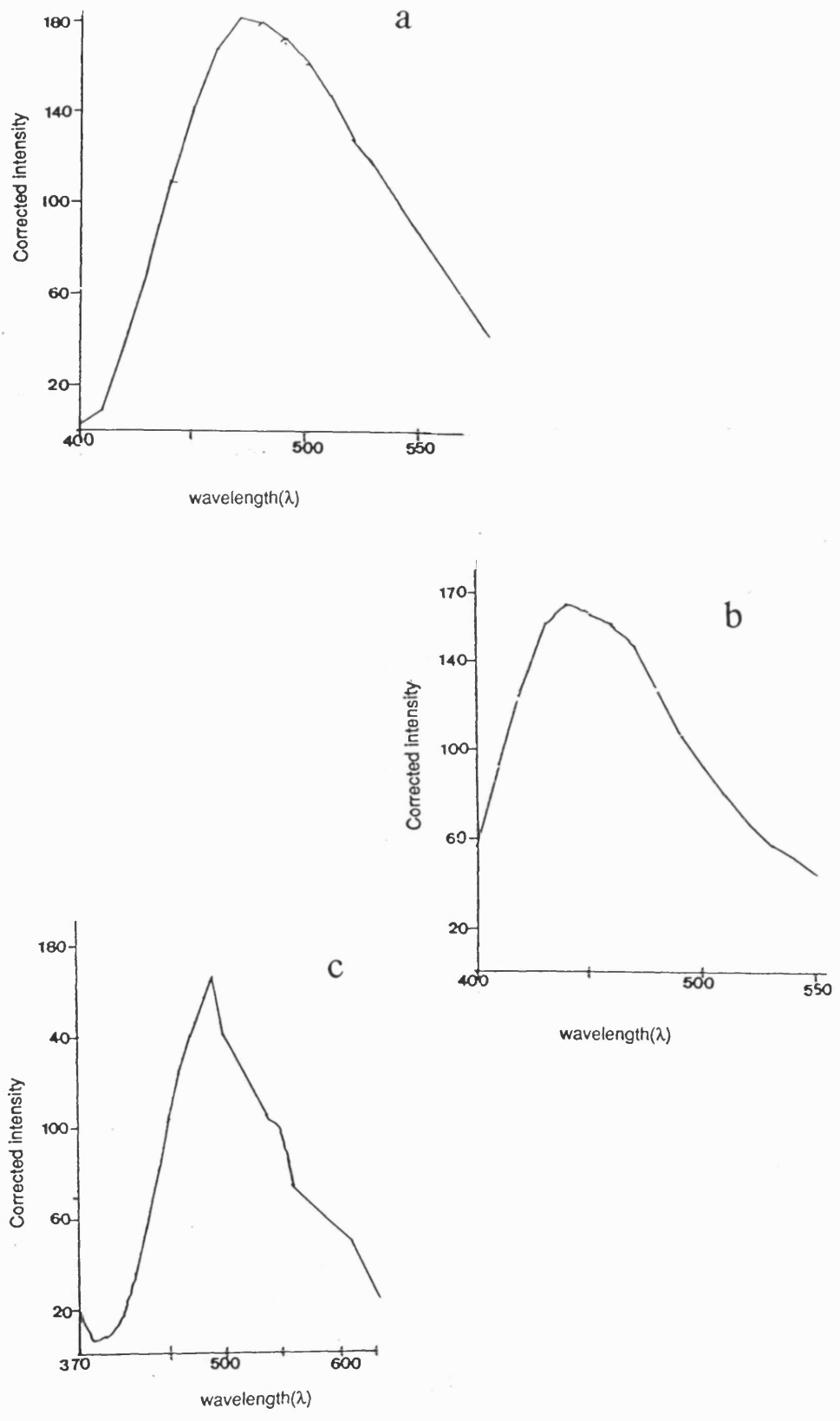


Fig 7.3 Corrected emission spectra (a) DAS, (b) Tinopal CBS-X , (c) Tinopal GS.

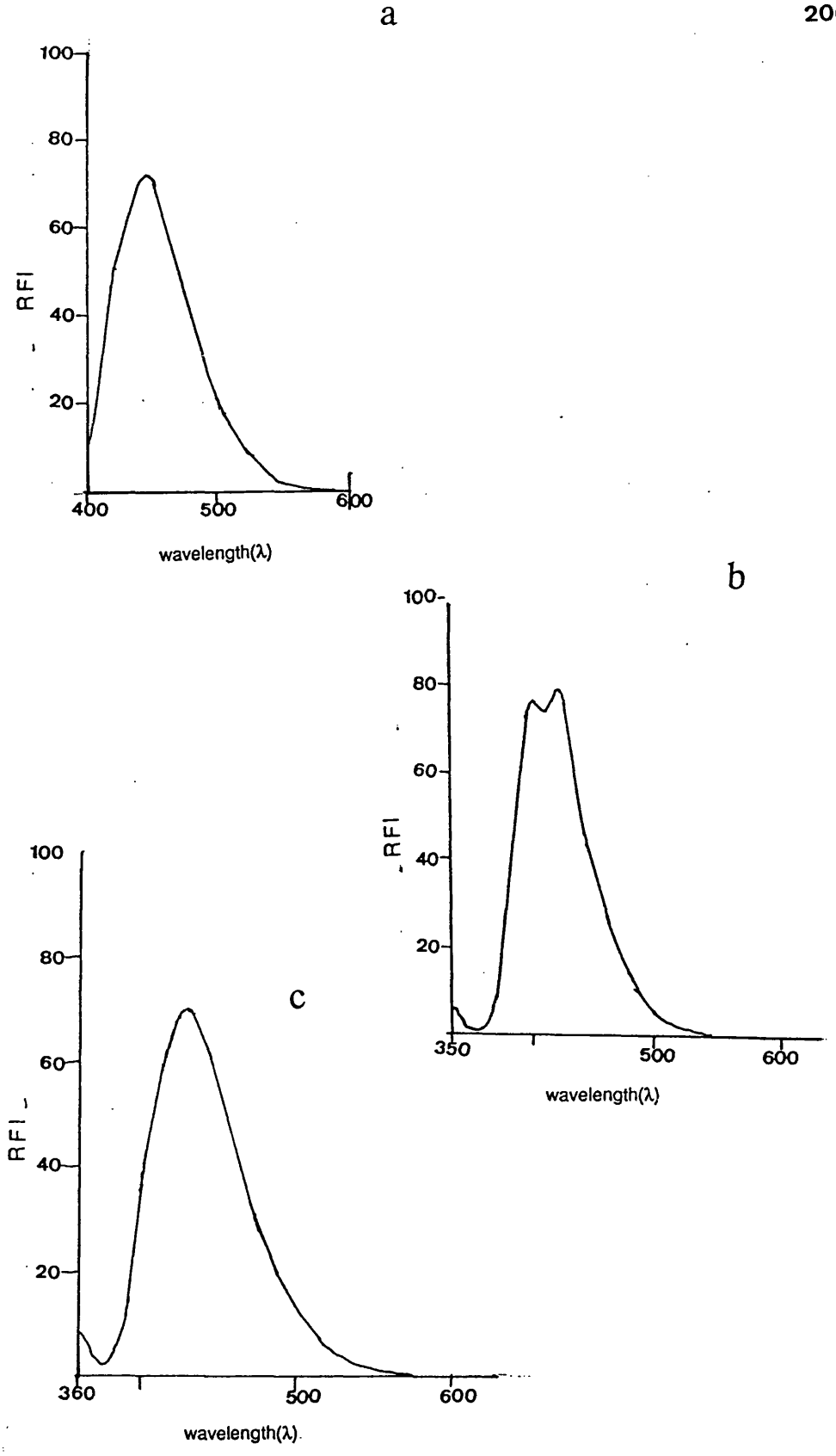


Fig 7.4 Apparent emission spectra of dye -TBA ion-pair, codes same as Fig 7.3.

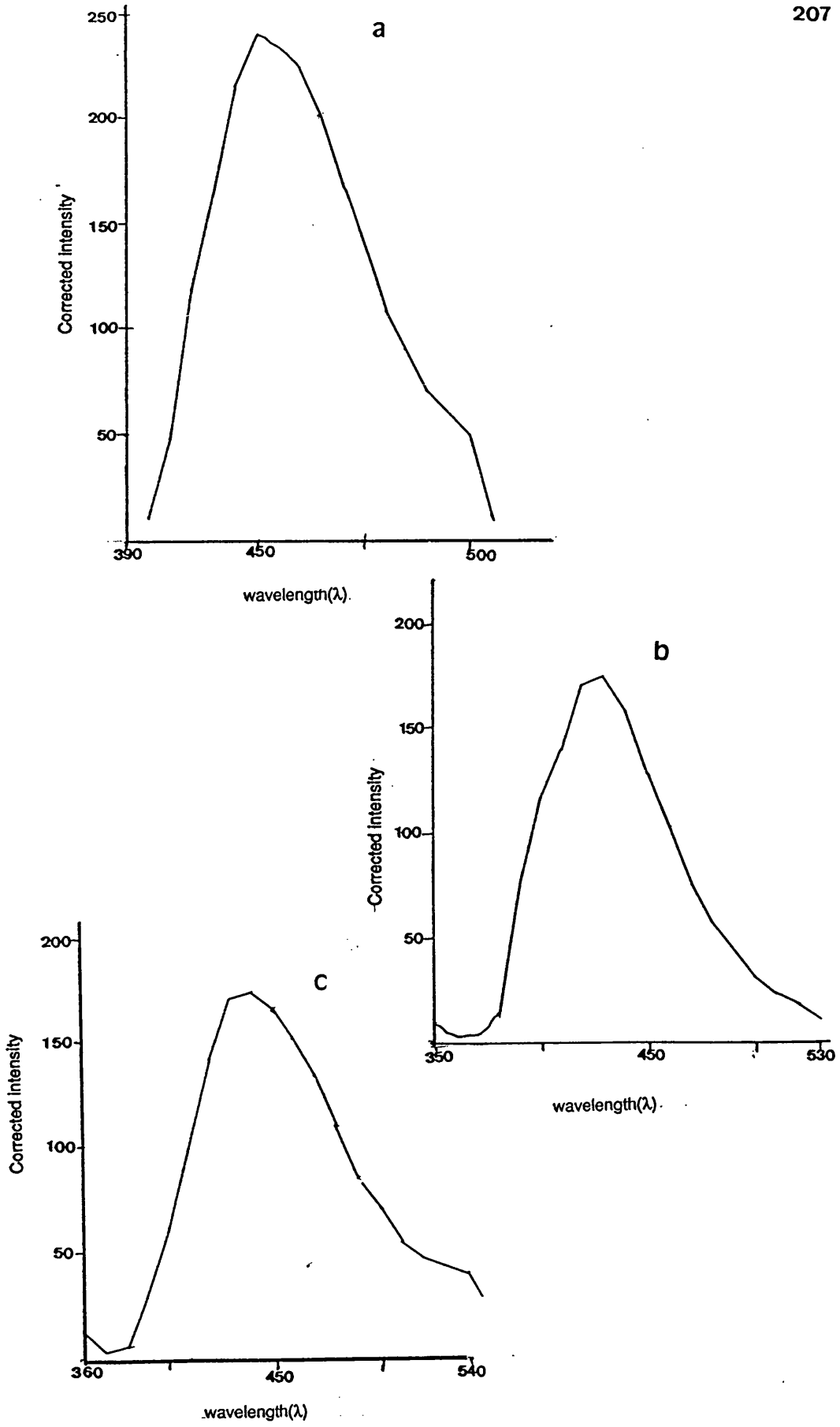


Fig 7.5 Corrected emission spectra of dye -TBA ion-pair, codes same as Fig 7.3

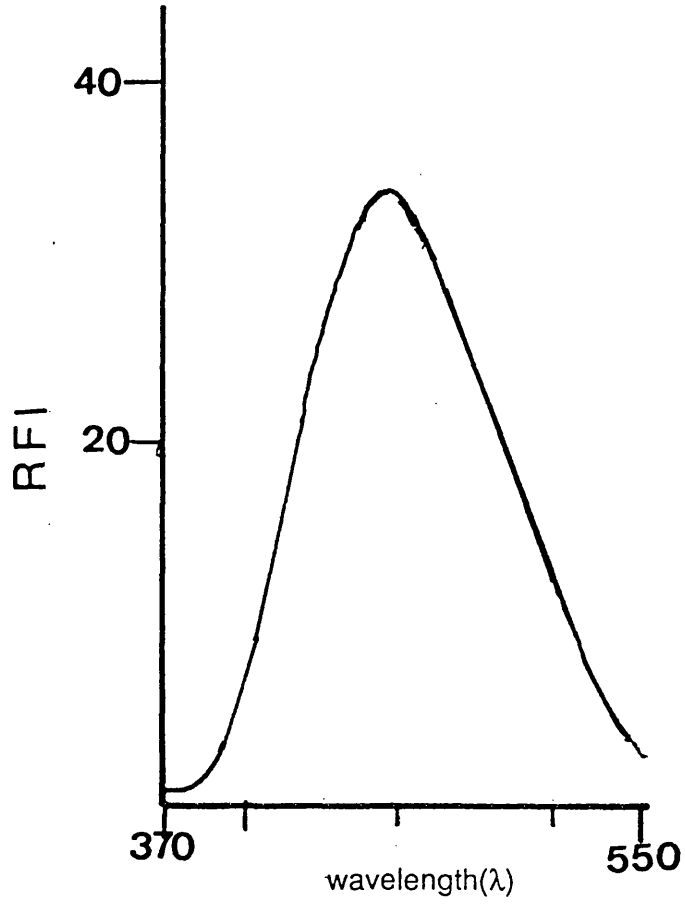


Fig 7.6 Emission spectrum of RBS-200

The fluorescent sensitivity index (FSI) is an important measure of how fluorescent a substance is. The greater the FSI value, the greater the fluorescence of that substance.

The optical data for DAS, CBS-X and Tinopal GS are shown in Table 7.5. DAS was used as a control to check that the experimental method that was followed gave results comparable with those obtained by Westerlund and Borg (1973). TABLE 7.5 shows that although the emission spectra were corrected in a different way to that used by Westerlund and Borg, the results obtained were in close agreement with their data. Initial attempts were made to measure the emission spectra of TBA-DAS ion-pair in methylene chloride but it was found that fluorescence dropped rapidly and was difficult to measure. It was finally found that after phase separation, if the organic solvent is diluted with ethanol, it was easier to measure the fluorescence spectra. This was also found by Tserng and Wagner (1976).

TABLE 7.5 Optical properties of fluorescent dyes.

Substance	Ex max (nm)	log ε	Em max (nm)	H (v')	Quantum yield φ	FSI = log εφ /H
DAS	383	3.80[a]	479	0.465 0.445[a]	0.465 0.36[a]	3.71 3.71[a]
CBS-X	348	4.75	432	0.48	0.49	4.75
GS	364	4.76	490	0.46	0.18	4.34
TBA-DAS	383	3.91 3.84[a]	450 452[a]	0.42 0.42[a]	0.55 0.69[a]	4.02 4.05[a]
TBA-CBS	348	4.71	430	0.40	0.64	4.91
TBA-TGS	364	4.70	490	0.46	0.49	4.77

[a] values obtained by Westerlund and Borg (1973) .

The CBS-X anion has a better fluorescence sensitivity (4.75) than DAS (3.71) and Tinopal GS (4.34) in water. In organic solvent the sensitivity of the CBS-X-TBA (4.91) ion-pair is also better than DAS-TBA ion pair (4.01) and Tinopal GS-TBA ion-pair(4.77). Tinopal RBS-200

optical properties are similar to Tinopal GS. The emission spectrum of Tinopal RBS 200 is shown in Fig 7.6.

7.7.3 Extraction Constants

As the FSI of the ion-pair CBS-X-TBA was better than DAS-TBA and TGS-TBA an attempt was made to determine its extraction constants. Initial attempts for the determination of extraction constants were made using flow injection analysis as described earlier in Methods for determination of extraction constants (7.4.1) using DAS as a control. Using batch extraction studies with an aqueous phase consisting of 0.1M NaH_2PO_4 and methylene chloride as an organic phase Westerlund and Borg (1973) had found the log E_{QX} for the DAS-TBA ion pair to be 5.85. For FIA, the aqueous and organic phases were the same as used by Westerlund and Borg (1973) except that the aqueous phase contained the fluorescent ion-pairing agent. Two studies were performed.

1. The concentration of the ion-pairing agent (DAS) C°_X was varied while keeping the concentration of TBA constant.($1X10^{-4}$ M).

2.The concentration of ion-pairing agent was kept constant while the concentration of the cation C°_Q was varied.

The absorbance of the TBA ion-pair was monitored at 383 nm.

TABLE 7.6 shows the results

TABLE 7.6

Exp	Conc $[C^{\circ}_X{}^-]$	Conc $[Q^+]$	$\frac{A_{max}}{A_{min}}$	intercept	slope	log E_{QX}
1 (A)	1-20 X 10^{-5}	1 X 10^{-4}	7.69	3.58	3.49 X 10^{-4}	4.01
(B)	5-250 X 10^{-6}	1 X 10^{-4}	9.72	3.92	1.96 X 10^{-4}	4.30
2	1 X 10^{-4}	2-25 X 10^{-6}	3.38	0.668	1.16 X 10^{-4}	3.75

Exp = experiment

The plots of 1/A vs 1/X (1/Q) gave straight lines in all cases as shown in Fig 7.7 . In Fig (A) 1/A is 1/Absorbance as used by Johansson et al, however in Fig (B) and (C) 1/A is 1/Peak areas. In practice measuring peak areas was found to be more accurate than noting absorbances at the peak maximum on a UV HPLC detector. As can be seen from the above Table there is very little difference between 1 (A) and (B) and so for ease of use peak areas were employed.

TABLE 7.7 shows the results when CBS-X was used instead of DAS as the ion-pairing agent, with the absorbance monitored at 348 nm.

TABLE 7.7

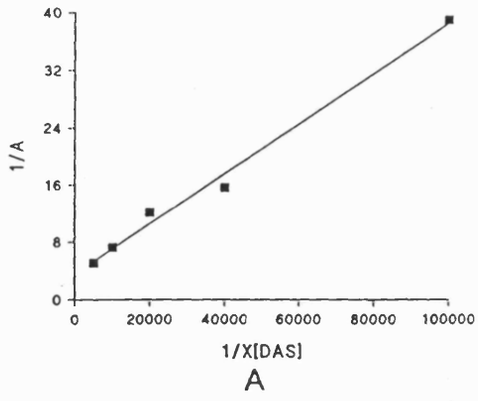
Exp	C^{o}_{X}	C^{o}_{Q}	$\frac{A_{max}}{A_{min}}$	Intercept	Slope	log E_{QX}
1	$1\text{-}20 \times 10^{-5}$	1×10^{-4}	3.39	7.72	2.2×10^{-5}	4.54
2	1×10^{-4}	$3\text{-}25 \times 10^{-6}$	13.77	-1.52	8.2×10^{-5}	n.d

n.d = not determined

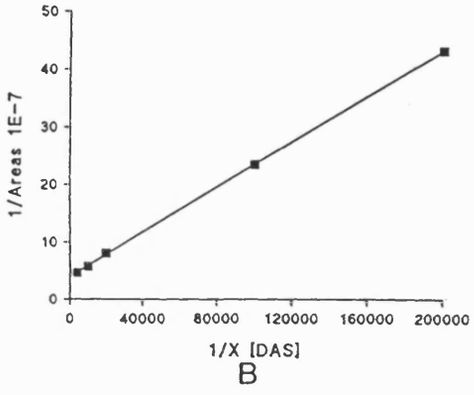
Experiments1 and 2 refer to the same conditions as described for TABLE 7.6. A plot of 1/A vs 1/X or 1/A vs 1/Q as shown in Fig 7.8 (a) and (b) gave a straight line in both cases However it was not possible to determine the extraction constant for experiment 2 because of the negative intercept.

The extraction constants obtained for DAS using FIA (see TABLE 7.6) in both experiments 1 and 2 are lower than those obtained by Westerlund and Borg i.e 5.85. Due to the lack of time, the reasons for the low results could not be fully investigated.

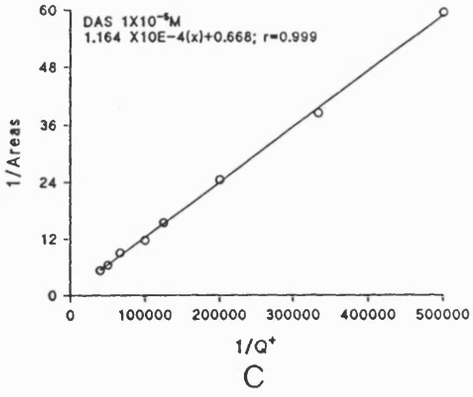
The calculated extraction constant for CBS-X i.e 4.54 (Experiment 1 TABLE 7.7) is very similar to that obtained for DAS (4.30) in this study. For experiment 2 when the concentration of cation C^{o}_{Q} was varied at a constant C^{o}_{X} a negative intercept was obtained. According to Johansson et al a plot of 1/A vs 1/Q will not give a straight line if side reactions are taking place e.g dissociation or dimerization of ion-pair in the organic



A



B



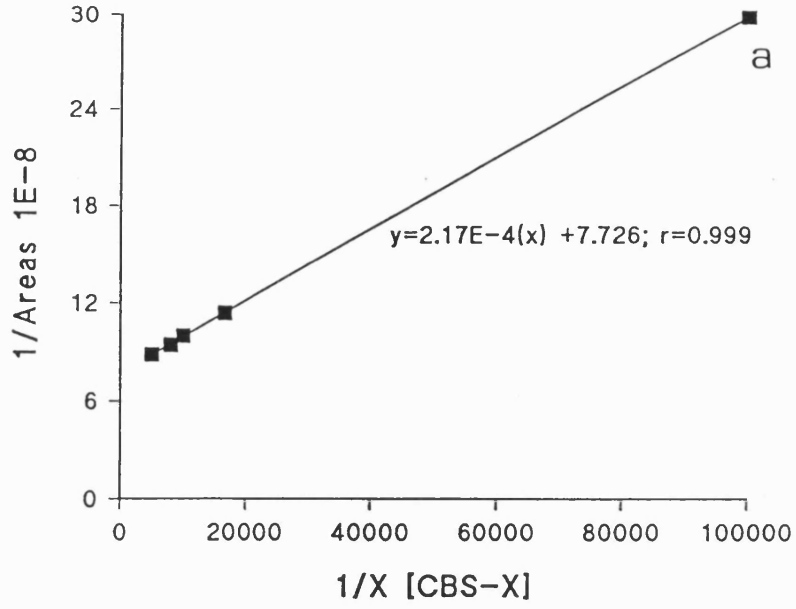
C

Fig 7.7 Determination of extraction constants for DAS using FIA, conditions as specified in the text.

(A)1/Absorbance vs 1/X at constant C^0_o, intercept 3.58; slope 3.49 X 10^{-4}; r=0.996.

(B) 1/Areas vs 1/X , intercept 3.92; slope 1.96 X 10^{-4} ; r=0.99.

(C) 1/Areas vs $1/Q^+$at constant C^0_x, intercept 0.668; slope 1.16 X 10^{-4}; r=0.99.



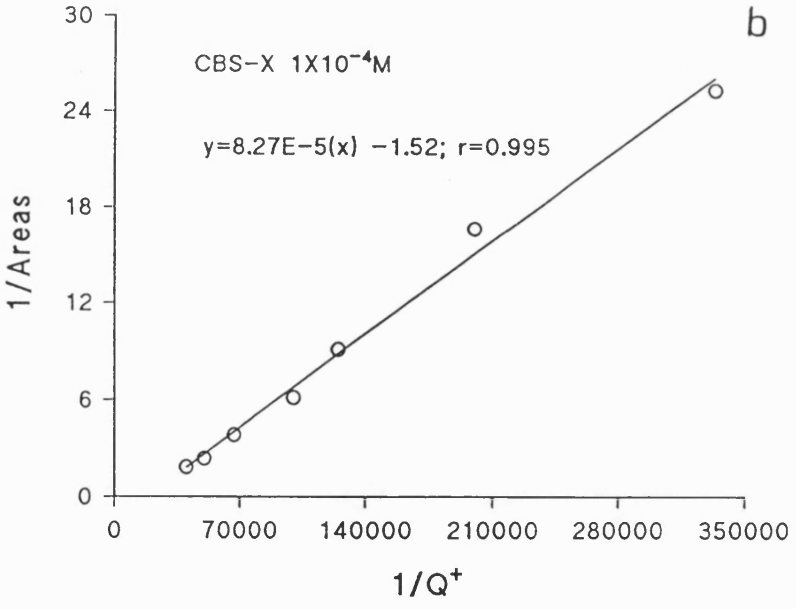


Fig 7.8 Determination of extraction constants for CBS-X, using FIA, conditions as described in the text.
(a) at constant C^0_Q: (b) at constant C^0_X

phase. One possible explanation for negative intercept could be the formation of 1+2 ion-pairs as CBS-X is a disulphonic acid.

For practical purposes, the ability of a fluorescent dye to act as an ion-pairing agent can best be judged by the sensitivity of the method and the background noise. The FIA system was connected to a fluorescence detector and the lowest concentration of cation was injected. The results are given below:

TABLE 7.8

Parameters	DAS	CBS-X
Aqueous phase	$1X\ 10^{-4}$	$1X10^{-5}$
Organic phase	DCM	DCM
Back ground	0	50 relative to DAS
Sensitivity 1 TBA $1X10^{-8}$ 2. TPA $1X10^{-8}$	Peak ht (n=3) 25 mm 17 mm	Peak ht 17 mm 5 mm

Batch extraction

DAS was used as a control to check the accuracy of the method so that the results obtained could be checked with the published ones. TABLE 7.9 shows the results of the partition experiment. The concentration of DAS was varied while the concentration of TBA was kept constant. For all the partition experiments the concentration of X was measured fluorometrically in the organic phase (using a standard curve)

TABLE 7.9

$C^{o}X^{-}$	log E_{QX} (n=3)	D_Q
$1X10^{-3}$	3.74±0.02	5.49
$1X10^{-4}$	4.50±0.01	3.16
$1X10^{-5}$	5.55±0.06	3.54
$1X10^{-6}$	6.67±0.01	4.67

The aqueous solution of DAS in 0.1 M NaH_2PO_4 was spiked with 2.5 X 10^{-5} M of TBA and methylene chloride was used as the organic solvent (5:5 mls). D_Q was calculated from the average value of E_{QX} from the relationship $D_Q = E_{Q\,X}[X^-]$.

CBS-X is a disulphonic acid, and initial experiments using 2.5 X 10^{-6}M TBA were quite inconclusive, so the concentration of TBA was raised to 5 X 10^{-6} M.

TABLE 7.10 shows the results of the partition experiment when the concentration of CBS-X was varied while the concentration of TBA was kept constant.

TABLE 7.10

$C^o\,X^-$	log E_{QX} (n=3)	D_Q
$1X10^{-3}$	2.02±0.02	0.104
$1X10^{-4}$	3.17±0.04	0.147
$1X10^{-5}$	4.23±0.01	0.169
$1X10^{-6}$	5.54±0.01	0.347
$1X10^{-7}$	5.80±0.01	0.063

D_Q was calculated from the average value of E_{QX}., where $D_Q = E_{QX}[X^-]$.

Keeping the CBS-X concentration constant at $1X10^{-5}$ M the concentration of TBA was varied from 1-10 X 10^{-6} M. As can be seen from the Fig 7.9 (a). The value of E_{QX} = 4.60.

Because CBS-X is a disulphonic acid and forms 1+2 ionpair complexes, its extraction constants are lower than that of DAS (5.85 as determined by Weterlund and Borg, 1970). The higher fluorescent sensitivity index is compromised by its ability to form 1+2 ion-pairs.

RBS-200 is structurally similar to Tinopal GS (a disulphonic acid), but like DAS is a monosulphonic acid and will form 1+1 ion-pairs. TABLE 7.11 shows the results of partition experiment with RBS-200 as an ion-pairing agent using the same conditions as used for DAS (see TABLE 7.9) i.e variable C^o_X and constant C^o_Q = 2.5 $X10^{-6}$ M.

TABLE 7.11

C^{o}_{X} M	log E_{QX} (n=3)	D_Q
$1X10^{-4}$	5.24± 0.27	17.37
$1X10^{-5}$	6.81± 0.01	64.56
$1X10^{-6}$	6.28 ± 0.24	1.90

D_Q was calculated from the average value of $E_{QX.}$ where $D_Q = E_{QX}[\ X^-]$.

Fig 7.9 (b) shows the plot when the concentration of RBS-200 was kept constant and the concentration of TBA was varied from 5-50 X 10^{-6} M. As can be seen from the plot the value of E_{QX} increases with increasing concentration of the ion-pair i.e at 50 X 10^{-7} M it is 7.69 and at $5X10^{-7}$ M it is 5.94.

According to Schill (1978) if the extraction constant changes with the concentration of the ion-pair components it indicates that the equilibrium is affected by dissociation or association process that involves the ion- pair or its components. Increase of extraction constant with increasing concentration of ion-pair components may indicate that under the experimental conditions used the ion pair is forming dimers or higher associates in the organic phase. Dimerization of QX is expressed by the dimerization constant:

$$K_{2\,(QX)} = \frac{[Q_2X_2]_{org}}{[QX]^2_{org}} \qquad \text{Eq 7.16}$$

Evidence of dimerization is obtained by measuring E_{QX} at constant C^{o}_{X} and variable C^{o}_{Q} as was done for Fig 7.9 (b). A plot of E_{QX} vs $[Q^+]$ must gives a straight line where the intercept gives the E_{QX} and $K_{2(QX)}$ is calculated from the slope, which in the case of Fig 7.9 are 5.79 and 0.0375 respectively.

The value of the extraction constant for RBS 200 (5.79) is very similar to the literature value for DAS 5.85, but the FSI of its TBA ion-pair are slightly better than DAS being 4.77, whilst DAS is 4.04. However the FSI of the DAS (anion) is 3.74 as compared to RBS (GS) 4.34, so RBS may give a slightly higher blanks.

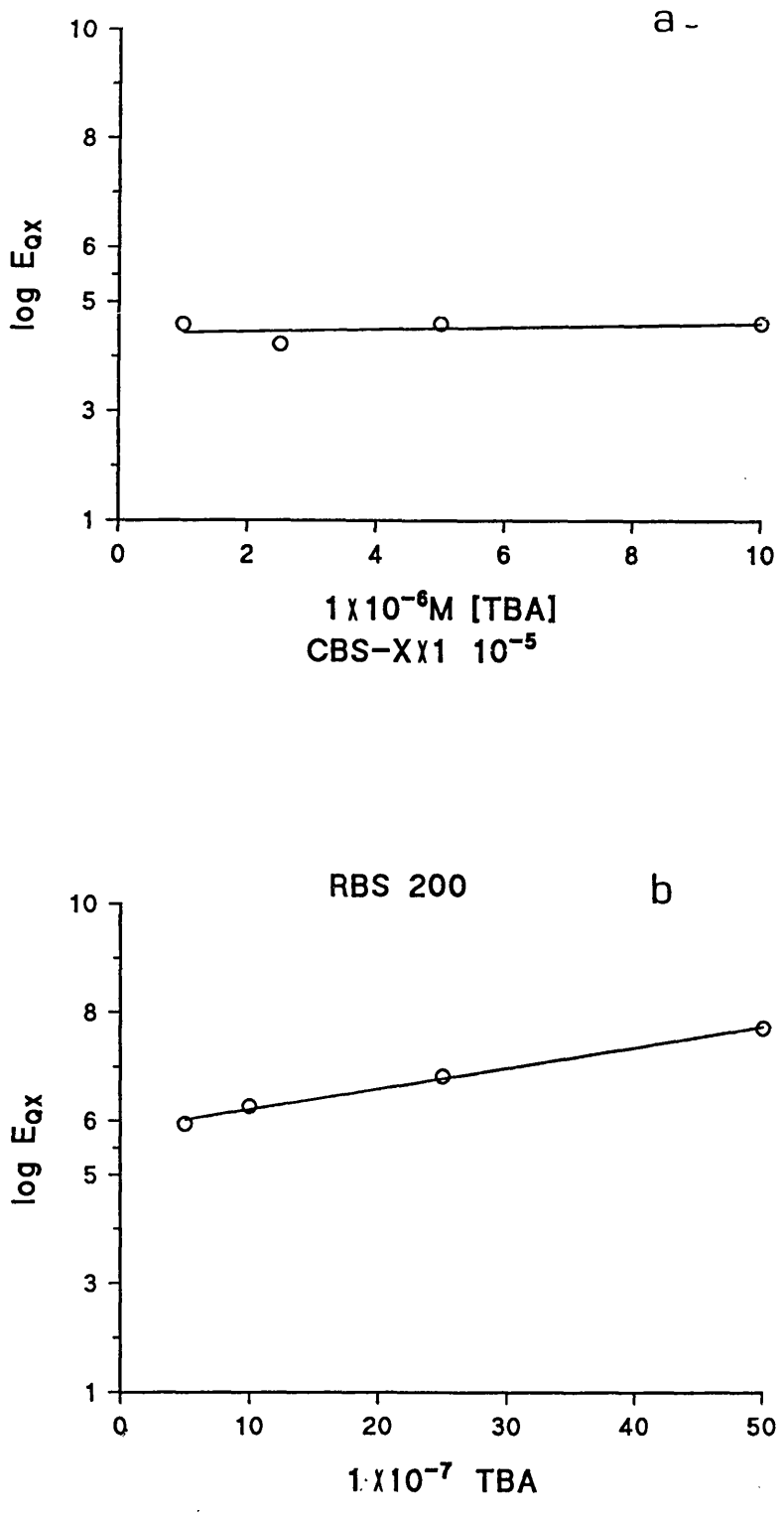


Fig 7.9 A plot of E_{ax} vs TBA concentration; (a) CBS-X, (b) RBS-200

7.9 Conclusions

In this section an attempt has been made to evaluate some fluorescent dyes used in detergents for use as post-column ion-pairing agents with respect to their optical properties and extraction constants. RBS 200 seems a promising candidate and may prove an alternative to DAS as a fluorescent ion-pairing agent .

Flow injection analysis when used for the determination of extraction constants for fluorescent dyes gave lower results than batch extraction studies. This may be because the equation used for determining extraction constants is based on absorbance which is suitable for high concentrations but is limited at low concentrations. On the other hand fluorescence is erratic at high concentration and measurements are made at lower concentrations where A< 0.01. Johansson et al (1980) had combined Eq 7.17 with Beers law (7.18) to obtain an equation which was used to calculate extraction constants using flow injection analysis (See Section 7.41 Eq 7.15).

$$D_{tot} = D_{pre}D_{post}\,(r+1(K_{ex(HBP)}[P^-]^{-1} \qquad \text{Eq.7.17}$$

$$A = \varepsilon l\,[HBP]_{cell} \qquad \text{Eq 7.18}$$

where:

$D_{tot} = C°_{HB}\,([HBP]_{cell})^{-1}$

$D_{pre} = C°_{HB}\,(C_{HBmix})^{-1}$ and

$D_{post} = [HBP]_{org}([HBP]cell)^{-1}$ and

$C_{HBmix} = C_{HB} = (HB^+) + r\,[HBP]_{org}$.

Perhaps a combination of equation 7.17 with the fluorescence equation i.e If = 2.3 φ.εcl (Eq 7.2) may give better results for the determination of extraction constants of fluorescence dyes using FIA.

Conclusions

A dual-channel phase separator designed by Dr T. M. Jefferies and made by SSI, PA, USA, has been evaluated for use as part of an HPLC post-column ion-pair extraction detector system. The phase separator routinely operated at a separation efficiency of 0.8 or more which ensured maximum peak heights and required very little maintainence during use. It can operate with HPLC eluents up to the point of miscibility with a selected organic phase. Band spreading was minimal i.e. 3 - 5 sec.

This work also demonstrated the use of gradient elution with post-column extraction detectors using narrow bore columns. A low pressure gradient system was modified for use with narrow bore columns to run gradients at low flow rates of around 0.4 ml min^{-1} by reducing the delay time from 12.5 minutes to 4.0 min resulting in reproducible gradients.

The post-column system was found to be more selective (Chapter 3 and Chapter 6 of this thesis) compared to low U.V for the detection of drugs and metabolites in biological matrices such as plasma and urine .

Cyano columns, although suitable for the chromatography of basic drugs with minimum tailing (Badiru and Jefferies 1988) were found to be relatively unstable. Suplex pKb -100 proved an efficient column for chromatography of basic drugs and could be used for longer periods with due care.

A post-column detection system has been developed for the detection of the two major cocaine metabolites in urine. i.e BE and EME with adequate sensitivities of 100-250 ng/ml^{-1} and is proposed as a general screening method for identification of cocaine abuse. This work also demonstrates the use of MTBSTFA as an HPLC pre-column non-labelling derivatization reagent.

A solid phase extraction method developed in this thesis for the extraction of cocaine metabolites from urine using large capacity (300 mg) cartridges has increased recovery of EME from 40% (Ortuno et al. 1990) to over 80%.

The examination of the optical properties and extraction constants of various fluorescent dyes examined in this thesis showed that RBS-200 (Ciba Giegy, Clayton, Manchester, U.K) has a potential as a post-column ion-pairing agent for detection of tertiary amines and quaternary ammonium compound and may be as good or slightly better than DAS.

Most of the work carried out in this thesis was done using a PE 203 spectrofluorometer with a 150 µl flow cell. The need for a proper HPLC fluorescence detector with a smaller flow cell was greatly felt as it would have greatly improved the chromatography as well as sensitivity.

Although SPE provides a cleaner sample than LLE, the presence of interfering peaks (see Chap 3, 5 and 6) from the Vac Elut system and the cartridges was quite frustrating. Some of the interfering peaks were the result of the eluent attacking the stainless steel needles of the Vac Elut system, others were due to the cartridges (see Chapter 5). The use of PTFE needles for the Vac Elut manifold would greatly improve things. Much work needs to be done by the manufacturers in collaboration with research institutions to gain all the advantages which SPE offers for sample preparation of drugs and metabolites from complex matrices such as biological samples.

Although in this work non-labelling pre-column derivatization techniques were used in combination with post-column ion-pair extraction detector, the combined use of non-labelling and labelling derivatization techniques would perhaps further increase the sensitivity of the method. For example, butylation of BE and derivatization of EME with 1-anthronyl nitrile.

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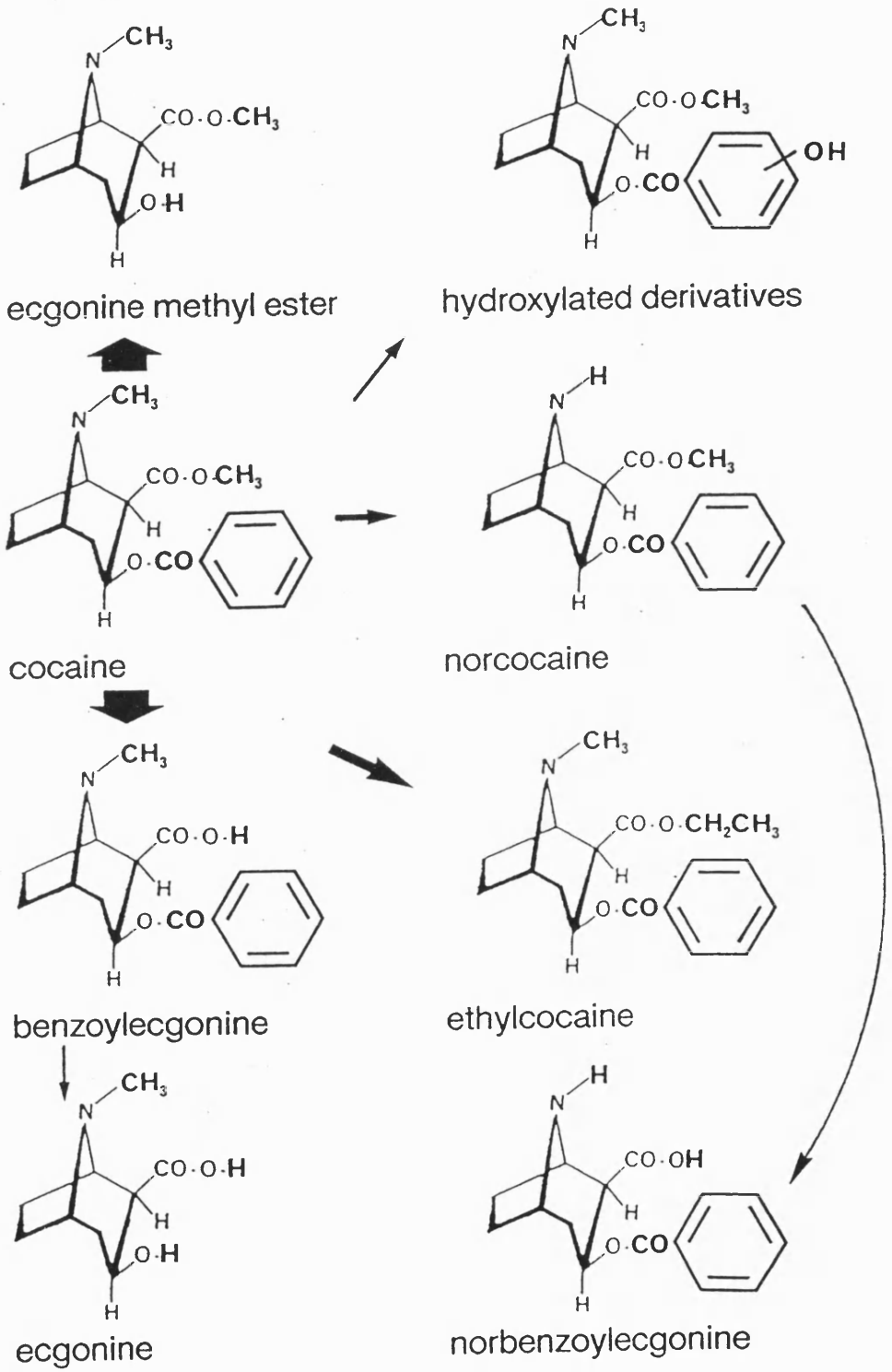
COCAINE METABOLITES



APPENDIX I

TABLE 1 A Approximate chemical shifts of important functional groups of cocaine metabolites as shown in Fig 1A - 6A

Fig	Compound	Aromatic	$-OCH_3$	$=NCH_3$
1 A	Cocaine	7.2-7.9	3.7	2.8
2 A	Ethylcocaine	7.2-7.9	1.2 & 4.2[a]	2.2*
3 A	Norcocaine	7.2-7.9	3.7	----
4 A	BE	7.2-7.9	----	2.8
5 A	Ecgonine	-----	-------	2.8
6A	EME	-------	3.7	2.8

[a] $-O-CH_2-CH_3$

* ethylcocaine base

BE= benzoylecgonine

EME = ecgonine methylester

------ not present

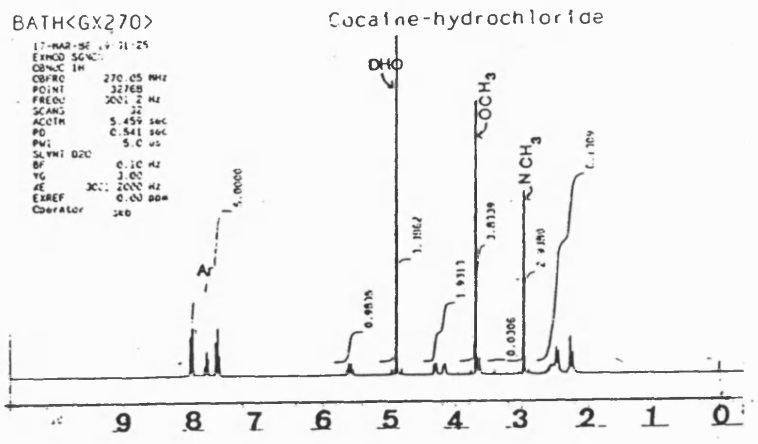


Fig 1A ^{1}H NMR spectrum of cocaine

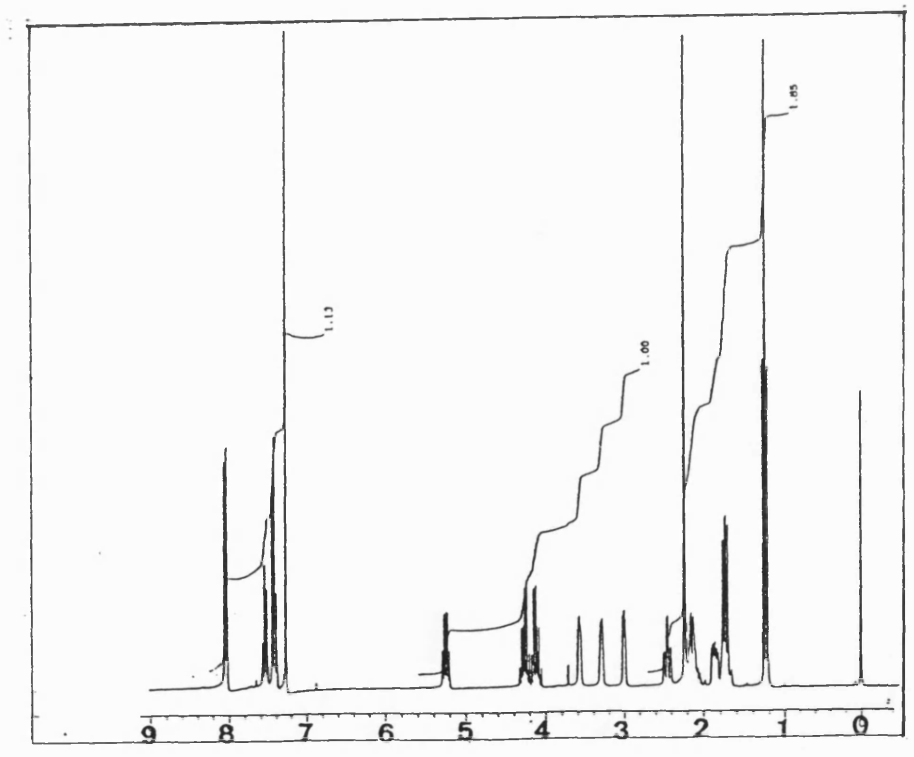


Fig 2A ^{1}H NMR spectrum of ethylcocaine

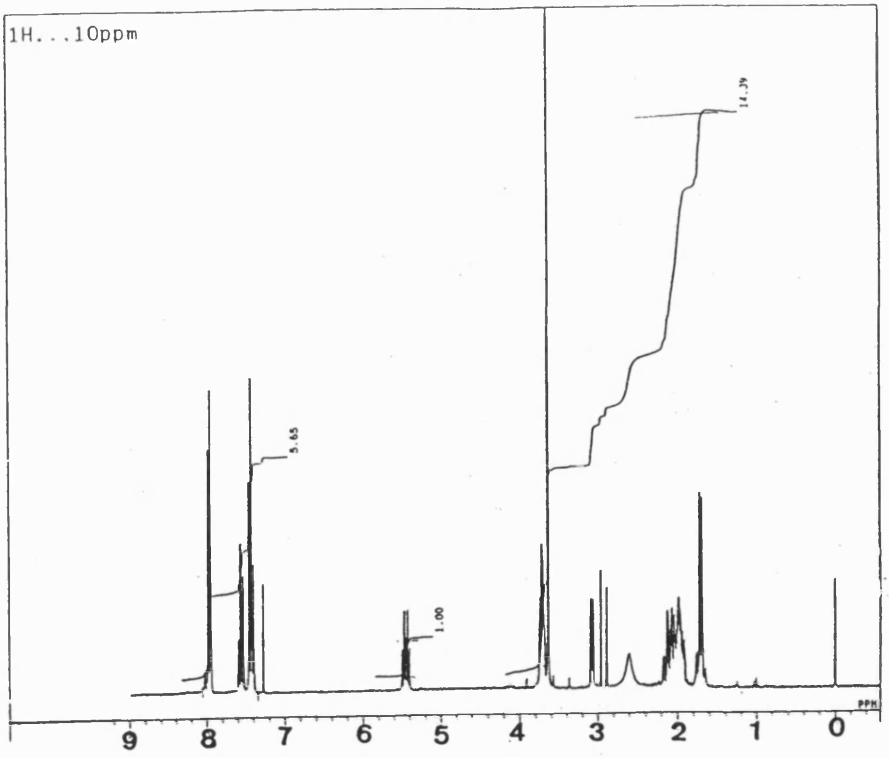


Fig 3A ^{1}H NMR spectrum of norcocaine

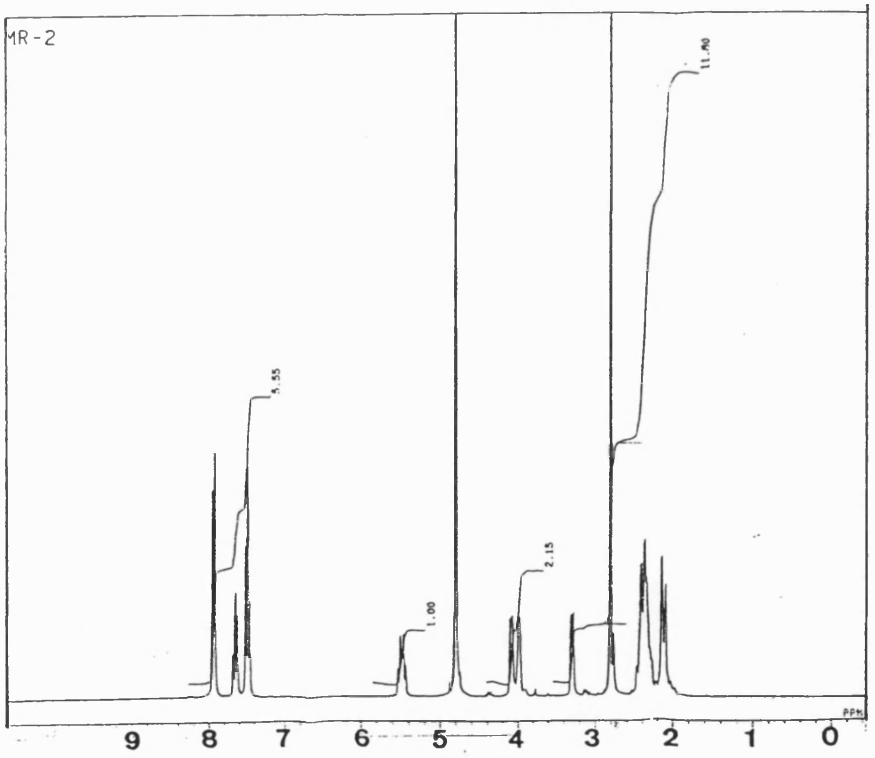


Fig 4 A ^{1}H NMR spectrum of benzoylecgonine

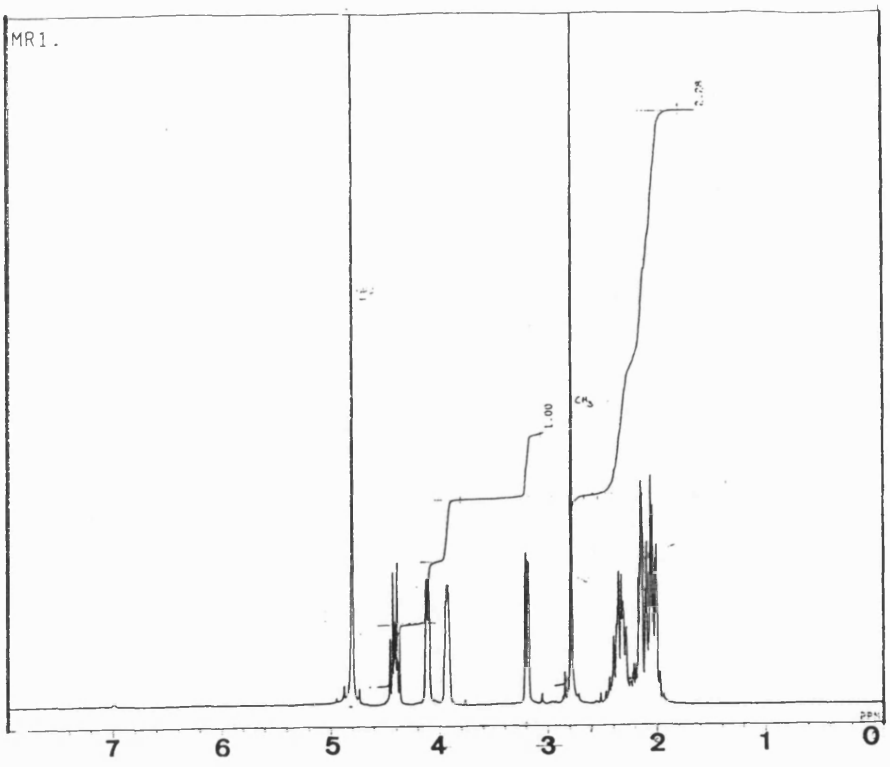


Fig 5A ^{1}H NMR spectrum of ecgonine

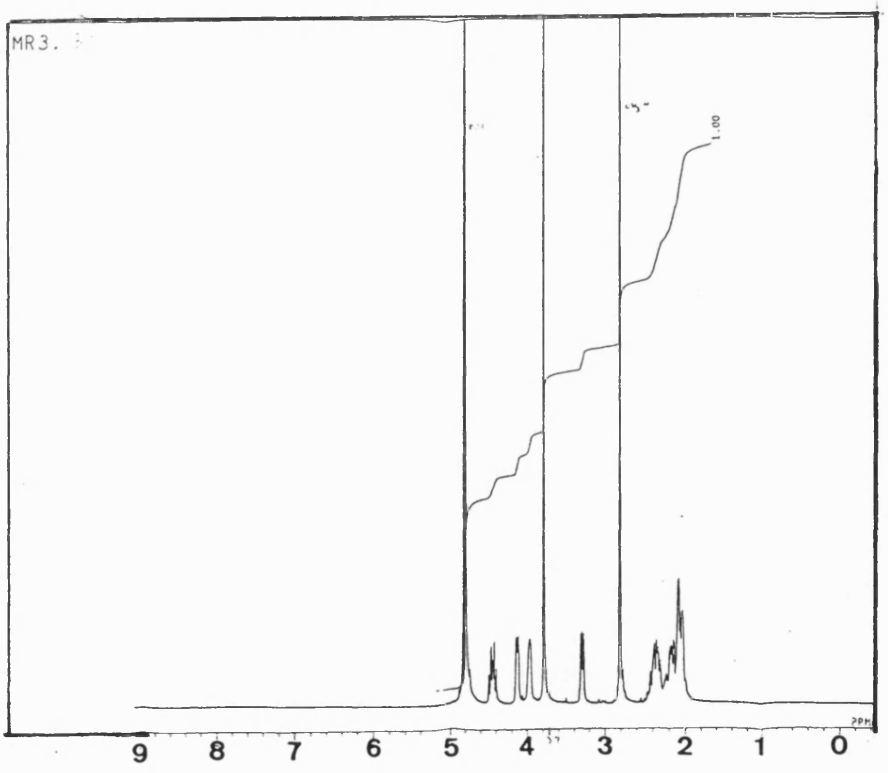
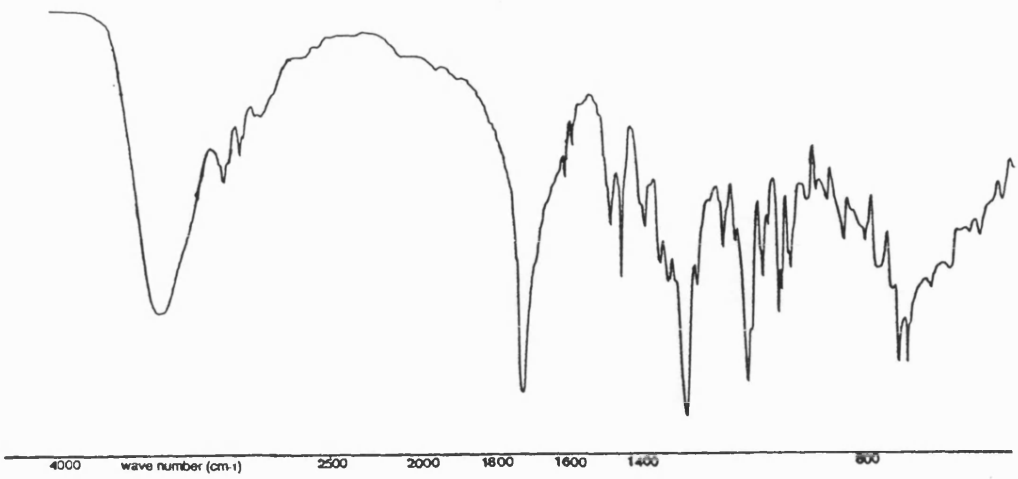
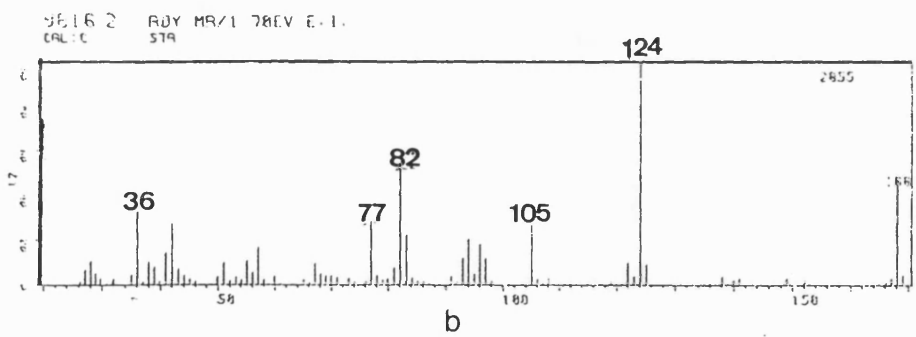


Fig 6 A ^{1}H spectrum of ecgonine methylester.



a



b

Fig 7 A (a) I.R Spectrum of BE, 1275, 1720, 1618, 717 (Clark)
(b) Mass spectrum of BE, m/z 124, 82, 168, 77, 105, 42,94, 83 (Clark)

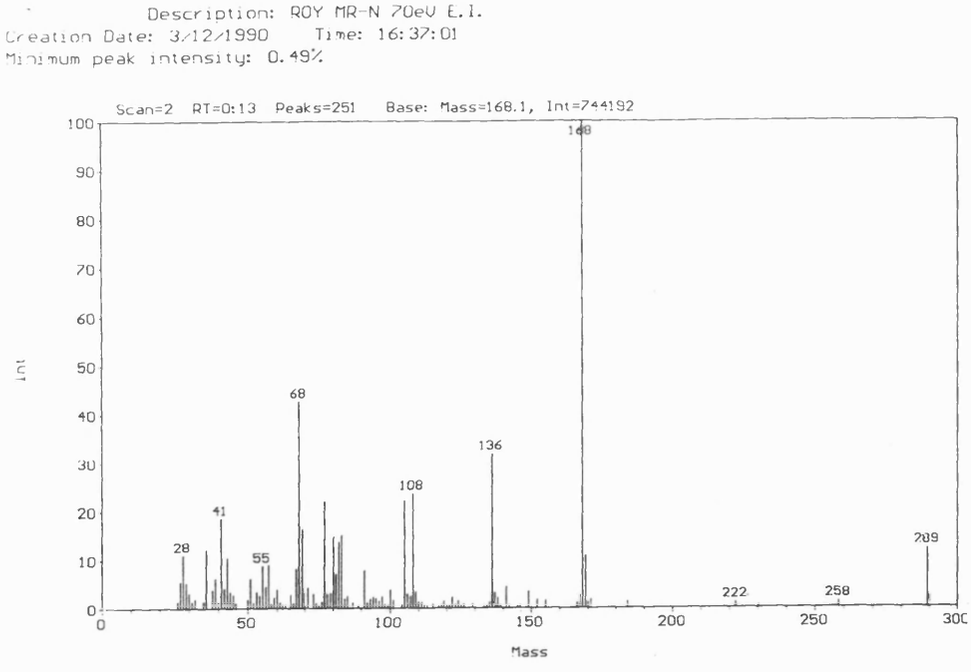


Fig 8 A Mass spectum of norcocaine, m/z 289, 168, 105, 69, 108, 136 (Labelle, M.J., Callahan, S.A., Latham, D.J., and Lauriault, G. (1988) Analyst, **113**, 1213-1215.

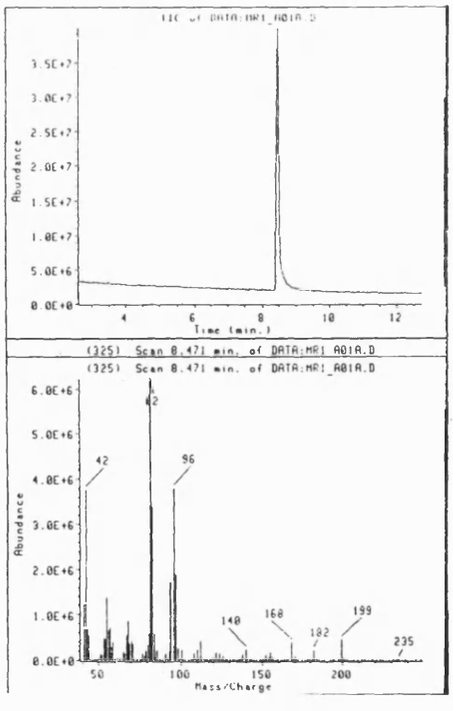


Fig 9 A GC-MS of ecgonine methylester, m/z 199, 82, 83, 96 (Ambre et al. 1982).

APPENDIX II

TABLE A 1 Fluorescence emission spectra of standards expressed in relative quanta units

A: Quinine sulphate 1µg/ml in 0.05 M H_2SO_4

B: 3- Aminophthalimide 1 µg/ml 0.05 M H_2SO_4

C: 2- Aminopyridine 1 X 10^{-5} in 0.1 N H_2SO_4, excitation at 285 nm

λ nm	A Q/Q max %	B Q/Q max %	C Q/Q max %
320	-	-	2.5
330	-	-	9.5
340	-	-	33.0
350	-	-	66.5
360	-	-	94.0
368	-	-	100.0
370	-	-	99.5
380	-	-	91.8
390	-	-	76.0
400	11.8	-	53.5
410	26.7	-	37.0
420	47.8	-	26.5
430	68.3	-	17.5
440	85.5	-	10.8
450	95.4	12.5	7.5
460	100.0	32.7	-
470	98.3	54.2	-
480	90.0	75.0	-
490	78.8	92.0	-
500	70.0	99.5	-
510	58.5	100.0	-
520	49.0	97.0	-
530	42.0	94.0	-
540	33.2	86.5	-
550	29.1	71.6	-
560	-	60.8	-
570	-	56.5	-
580	-	40.0	-
590	-	34.0	-
600	-	27.0	-
610	-	16.6	-
620	-	13.5	-
630	-	10.4	-

Q/Q_{max}: Quanta reading divided by quanta maximum at the same concentration.

Data taken from White and Argauer (1970) p 49

TABLE A 2 Corrected emission spectrum of DAS at 348 nm

λ nm	Shimadzu	C.F*	Corrected readings
400	1.20	0.6356	1.88
410	4.93	0.532	9.26
420	17.91	0.462	38.76
430	28.95	0.413	70.10
440	40.29	0.37	109.0
450	47.16	0.3344	141.0
460	49.40	0.294	168.0
470	46.86	0.2574	182.10
480	40.89	0.228	179.5
490	34.93	0.203	172.0
500	28.06	0.1728	162.4
510	22.38	0.152	147.2
520	16.11	0.126	127.4
530	11.04	0.0952	116.0
540	6.86	0.0693	99.1
550	4.48	0.048	86.9

C.F correction factors obtained from TABLE 7.1-7.3

TABLE A 3 Corrected emission spectrum of Tinopal GS at 360 nm

λ nm	Shimadzu	C.F*	Corrected readings
350	14.02	0.725	19.35
360	31.64	0.585	54.08
370	10.74	0.549	19.57
380	2.98	0.501	5.96
390	3.58	0.467	7.67
400	6.56	0..6356	10.33
410	11.04	0.5318	20.76
420	16.71	0.462	36.18
430	23.28	0.413	56.38
440	29.85	0.370	80.76
450	34.62	0.334	103.54
460	36.53	0.294	124.37
470	36.11	0.257	140.32
480	33.73	0.228	148.07
490	29.85	0.203	148.08
500	28.65	0.173	165.83
510	21.19	0.152	139.39
520	16.71	0.126	132.14
530	11.64	0.095	122.28
540	7.16	0.069	103.82
550	4.77	0.048	99.37
560	4.77	0.064	74.62
570	2.68	0.039	68.01

contd-

l nm	Shimadzu	C.F*	Correction Values
580	2.80	0.038	54.73
590	1.79	0.030	58.72
600	1.19	0.022	54.27
610	0.895	0.018	49.75
620	0.895	0.022	40.70
630	0.656	0.028	23.45

The fluorescence readings of Shimadzu were measured from the chart recorder.

TABLE A 4 Emission spectrum of DAS-TBA

λ nm	Shimadzu	C.F*	Correction values
400	7.16	0.635	11.27
410	26.28	0.5318	49.39
420	55.0	0.462	118.88
430	68.05	0.4129	164.83
440	80.0	0.370	216.445
450	80.60	0.334	241.01
460	69.25	0.294	235.551
470	57.90	0.257	224
480	45.37	0.228	199.175
490	34.02	0.203	167.631
500	24.48	0.173	141.65
510	16.71	0.152	109.90
520	11.34	0.126	89.67
530	6.57	0.095	68.98
540	4.18	0.069	60.30
550	2.40	0.048	49.64
560	1.50	0.128	11.70
570	0.71	0.079	9.03
580	0.597	0.075	7.96
590	0.24	0.061	3.99
600	0	0.044	0.0

TABLE A 5 Emission Spectra of CBS-X-TBA ion-pair

λ nm	Shimadzu	C.F	Correction Values
350	6.56	0.725	9.05
360	1.80	0.585	3.06
370	1.80	0.549	3.26
380	7.76	0.501	15.50
390	35.82	0.467	76.70
400	74.62	0.635	117.40
410	74.02	0.532	139.20
420	79.04	0.462	171.86
430	72.23	0.413	174.95
440	58.50	0.370	158.30
450	42.39	0.344	126.76
460	30.48	0.294	103.56
470	19.70	0.257	76.54
480	13.13	0.228	57.65
490	8.96	0.203	44.11
500	5.38	0.173	31.09
510	3.58	0.152	23.55
520	2.38	0.126	18.87
530	1.19	0.095	12.54
540	0.597	0.069	8.61

TABLE A 6 Emission spectrum of Tinopal GS-TBA ion-pair

λ nm	Shimadzu	C.F *	Correction Values
360	8.35	0.585	14.28
370	3.0	0.549	5.48
380	3.60	0.501	7.15
390	14.32	0.467	30.68
400	38.20	0.636	60.11
410	53.13	0.532	99.91
420	65.67	0.462	142.14
430	69.85	0.413	169.17
440	63.88	0.370	172.83
450	55.52	0.334	166.032
460	44.77	0.294	152.30
470	34.62	0.257	134.52
480	25.0	0.228	110.07
490	17.31	0.203	85.28
500	12.53	0.173	72.55
510	8.35	0.152	54.95
520	5.97	0.126	47.20
530	4.18	0.095	44.0
540	2.87	0.069	41.35
550	1.20	0.048	24.82
560	0.58	0.128	4.68

LIST OF PAPERS PUBLISHED

1. I.M.Roy and T.M.Jefferies. Performance evaluation of an aqueous-organic phase separator for post-column reaction in high performance liquid chromatography and its applications to some basic drugs of abuse. **J. Pharm. Biomed. Anal. 8 (8-12) (1990) 831-835.**

2.I.M.Roy, T.M.Jefferies, G.H.Dewar and M.D. Threadgill. Analysis of cocaine, benzoylecgonine, ecgonine methylester, ethylcocaine and norcocaine in human urine using HPLC with post-column ion-pair extraction with fluorescence detection. **J. Pharm. Biomed. Anal. 10-12 (1992)** in press.